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(71) Applicant: THE UNIVERSITY OF TENNESSEE RESEARCH CORPORATION [US/US]; Research Corporation, 415 Communications Building, Knoxville, TN 37996 (US).

(72) Inventors: DALE, James, B. ; 72 Lombardy Road, Memphis, TN 38111 (US). LEDERER, James, W., Jr. ; 1680 Hayne Street, Memphis, TN 38119 (US).

(74) Agents: WEISER, Gerard, J. et al.; Weiser & Associates, 230 South 15th Street, Philadelphia, PA 19102 (US).

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(54) Title: RECOMBINANT MULTIVALENT M PROTEIN VACCINE

(57) Abstract

The invention relates to DNA sequences encoding epitopes which are used in recombinant multivalent hybrid protein vaccines to control group A streptococcal infections of multiple serotypes which may result in rheumatic fever.

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RECOMBINANT MULTIVALENT M PROTEIN VACCINE

FIELD OF THE INVENTION

The invention relates to recombinant multivalent M protein vaccines useful to control group A streptococcal infections of different serotypes which may result in rheumatic fever, rheumatic heart disease, and to other embodiments further described herein.

BACKGROUND OF THE INVENTION

Acute rheumatic fever (ARF) is the major cause of heart disease in children around the world. The disease is rampant in developing countries where prevalence rates of rheumatic heart disease may be as high as 35-40 per thousand individuals. By one estimate, it affects nearly six millon school-age children in India. Although the incidence of ARF in the United States and other Western countries declined markedly during the later half of the twentieth century, there has been a recent remarkable resurgence of the disease in the United States. Hence, the need for a safe and effective vaccine is urgent and serious.

Streptococci are a group of bacteria with the capacity to grow in chains. Many varieties are part of the normal bacterial flora in humans and are not especially harmful. However, a particular subgroup of streptococcal bacteria, called group A and represented by Streptococcus pyogenes, is a human pathogen. Between 20 and 30 millon cases of group A streptococcal infections occur every year in the United States alone. These cases include infections of the skin and throat, forms of pneumonia and a more recently identified disease resembling toxic shock. The most common infection is acute streptococcal pharyngitis, or strep throat, which occurs predominantly in school-age children. Strep throat qualifies as a major worldwide health problem if judged only by time lost from school and work and by the amount spent on related doctor's fees.

Strep throat's toll is much greater, however. In as many as 4% of the pharyngitis cases that are untreated or treated ineffectively, the strep infection leads to ARF. Current attempts to

revent ARF rely on treatment of the pharyngitis with antibiotics. During a recent outbreak of ARF in Utah, only a fourth of the patients sought health care prior to the onset of symptoms, and only a third recalled a recent sore throat. The finding that ARF may follow a subclinical infection in such a high percentage of individuals and the fact that access to health care in developing countries is not widely available serve to underscore the need for a safe and effective vaccine against group A streptococci.

The causal relationship between streptococcal pharyngitis and ARF was established over 50 years ago, yet the mechanism of the pathogenesis of the disease remains unclear. It is widely held that ARF is an autoimmune disease, and that in the susceptible host the infection triggers an immune response that leads to inflammatory and sometimes destructive changes in target tissues. Streptococci have been shown to contain antigens that are immunologically cross-reactive with host tissues and heart-cross-reactive antibodies from patients with rheumatic fever have been shown to react with streptococci. .However, it was also shown that sera from patients with uncomplicated pharyngitis also may contain heart-cross-reactive antibodies, yet these patients do not develop clinical evidence of carditis. Until the significance of tissue-cross-reactive antibodies in the pathogenesis of ARF is better understood, there remains a need to exclude potentially harmful epitopes from vaccine preparations.

The surface M protein of group A streptococci is the major virulence factor and protective antigen of these organisms. group A streptococci have developed a system for avoiding some of the antimicrobial defenses of a human host. Strains of streptococci that are rich in M protein evade phagocytosis by PMNs and multiply in non-immune blood. Yet, resistance to an infection by these bacteria is possible if the host's body can produce opsonic antibodies directed against the M protein. Such antibodies will neutralize the protective capacity of the M protein and allow the streptococcus to be engulfed and destroyed by phagocytes. The development of secretory or mucosal antibodies as opposed to serum opsonic antibodies, are also now suspected of playing an important role in preventing streptococcal infections.

A major obstacle to effective vaccine development has been the very large number of M protein serotypes. See, Stollerman, "Rheumatic Fever and Streptococcal Infection, Grune & Stratton (1975). These are reported to number about 82 to date and more can be expected to be identified.

It has been shown that antibodies against one serotype do not necessarily offer protection against others although some do cross-react with others. Immunity then appears to be type or sero-specific and optimal vaccines would require that most of the serotypes be represented. The concept of "rheumatogenic" and "non-rheumatogenic" organisms is supported by multiple surveillance studies over many years and in diverse areas of the world. Thus, there are probably about 12-15 serotypes responsible for most cases of ARF. Some of these are types 1, 3, 5, 6, 14, 18, 19, 24, 27 and 29.

To assist in a better understanding of the invention, a description of the M protein structure is useful. See, Scientific American, June 1991, Streptococcal M Protein by Vincent A. Fischetti. Considering a typical M protein structure such as that of type M6, approximately 80 percent of the M6 molecule is made of four distinct regions, each of which consists of repeated sequences of amino acids. These regions are arbitrarily designated by the letters A through D. Near the N-terminal, or amino end, the part of the molecule farthest from the bacterial cell, lies region A. This region has five tandem repeats, or blocks, of 14 amino acids each. The three central repeats are identical, whereas the repeats at each end of the region diverge slightly from the common amino acid sequence. Next on the molecule is region B, which has a similar five-repeat structure except that the repeated blocks contain 25 amino acids. Region C consists of two and a half tandem repeats of 42 amino acids each; these blocks are not as identical to one another as those in the A and B repeats. Region D is composed of four partial repeats containing seven amino acids. The section buried in the cell extends from about the last repeat of the C region to the C-terminus.

Adjacent to the D-repeat blocks is a non-repeat region containing an abundance of proline and glycine amino acids, which are distributed in a nearly regular pattern. Beyond that region

lies the C-terminal, or carboxyl, end of the molecule, which is the part within the cell. Near the C-terminal end are 20 hydrophobic amino acids and, at the terminus, six charged amino acids.

Similar arrangements of repeat blocks occur in the M proteins from type 5, 12, 24 and other streptococci. An alignment of the amino acid sequences of these different M proteins reveals that their C-terminal ends are more than 98 percent identical. Closer to the N-terminus, however, differences in sequence among M proteins increase. Consequently, the A-repeat blocks and a short amino acid region of about 10 to about 20 amino acids at the N-terminus are unique for each M molecule. This uniqueness is the major determinant of the sero-specificity of the immunological response.

In the amino acid sequence of M6 and later discovered in other M protein, another intriguing structural detail revealed itself. Running throughout all the repeat regions is an unusual seven-amino acid pattern: the amino acids in the first and fourth positions are hydrophobic; the intervening amino acids allow the protein to twist itself into a spiral shape called an alpha helix.

The seven-unit pattern in the arrangement of the amino acids in M6 indicates that the repeat regions of the protein molecule make up a long helical rod. The pattern in M6 is not perfect, nor is that pattern found in many other coiled-coil structures. Such irregularities probably account for the flexibility of the M molecules observed in electron micrographs. More important, the characteristics of these irregularities differ in the A-, B- and C-repeat regions. This observation suggests that each repeat region evolved independently and may have a distinct function. For an illustration of the protein sequence of M6 determined by cloning its gene, and for different forms of related M proteins when mutant streptococci delete copies of the amino acid repeats found in the parental molecule, especially in the N-terminus, see Scientific American, cited above. Studies have shown that each M protein fiber on a streptococcal cell wall is about 50 to 60 billionths of a meter long and consists of a single coiled-coil dimer (two M proteins coiled around each other).

It is likely that M proteins of all serotypes are built along a basic theme; they have a lengthy coiled-coil rod region in their centers that is flanked by a floppy section at the N-terminal

end and an anchoring region at the C-terminal end. Because the alpha-helical coiled-coil structure can accommodate a large number of varying amino acid sequences, many different M proteins with the same general conformation can be constructed, as is shown hereinafter.

For an M protein to protect a streptococcus, it must be able to attach to the organism. The mechanism that holds surface proteins on gram-positive bacteria is still poorly understood, but various studies of the M protein have been enlightening in that respect.

It is believed that the 20-hydrophobic amino acids near the C-terminal end are positioned into the similarly hydrophobic membrane itself, whereas the charged amino acids at the very terminus protruded into the aqueous cytoplasm. Because the charged amino acids would resist moving into a hydrophobic environment, they would act like a knot at the end of a string, preventing the M molecule from being pulled through the membrane. That mechanism may be valuable for some proteins attached to membranes. More recent evidence indicates, however, that the attachment mechanism for M protein and other bacterial surface proteins may actually be more sophisticated. Studies have revealed that all surface proteins from gram-positive bacteria have a similar arrangement of hydrophobic and charged amino acids at their C-terminal end. See for instance Fischetti et al., Surface Proteins from Gram-Positive Cocci Share Unique Structural Features, New Perspectives on Streptococci and Streptococcal Infections, (G. Orefici, Editor), Gustav and Jena (Publishers) 1992.

More important, however, a short six-amino acid sequence adjacent to the hydrophobic region is highly conserved in all the known surface proteins of gram-positive bacteria. The sequence consists of a leucine, a proline, a serine, a threonine, a glycine and a glutamic acid. Its designation is usually abbreviated as LPSTGE.

The importance of the LPSTGE sequence in the attachment of the M protein (and probably in all other proteins with this sequence motif) was shown by reported genetic experiments. It was found that if only the LPSTGE sequence is removed from the M protein gene, the M molecule that was produced would not attach to the bacterial membrane. This result suggested that the

hydrophobic domain and the charged amino acids at the C-terminus are not sufficient for membrane attachment and that the LPSTGE motif may be an important signal for initiating the process.

In nearly all surface proteins found in gram-positive bacteria, there is another distinctive region that spans about 50 to 75 amino acids on the N-terminal side of the hydrophobic region. This part is probably located within the peptidoglycan. Proline, glycine, threonine and serine constitute a high percentage of these amino acids. The reason for their prevalence has not been fully explored, but it is thought that prolines and glycines can create turns and bends in proteins. One hypothesis holds that cross-links in the peptidoglycan can weave through the proline- and glycine-induced bends, thereby stabilizing the M protein's position in the cell wall.

The knowledge that all known surface proteins on gram-positive bacteria attach themselves by a similar mechanism may open new avenues, such as controlling infections caused by these organisms. Surface proteins help pathogenic organisms initiate infections. It has been proposed that by preventing the proteins from anchoring to the bacterial cell, one should eventually be able to block infections and circumvent some of the problems associated with resistance to antibiotic therapies.

Just as the structures at the C-terminal end of the molecule provide information on how the M protein attaches to the bacterial cell, structures at the N-terminal end offer clues about how the molecule helps to fend off phagocytes. The N-terminal end of all M molecules has an excess of negatively charged amino acids, which results in a net negative charge for the region. Mammalian cells also exhibit a net negative charge on their surface. It has been suggested that the charge on M proteins may thus have evolved to hamper contact between streptococci and phagocytic cells through electrostatic repulsion. It has been proposed that one function of the central rod in the M protein is to act as a shaft for holding the negatively charged N-terminal end - and phagocytes - away from the bacterial surface.

At the N-terminal end of the coiled-coil rod, there is also a hypervariable region. This part of the molecule has a distinctive sequence in each M serotype. The hypervariable region consists

of the short 10-30-amino acid non-helical sequence and if present, the adjoining A-repeat region. The hypervariable region plays an important role in the biological activity of the molecule; antibodies against this area are optimal at promoting phagocytosis and killing of the streptococci. This observation again explains why only serotype-specific antibodies protect against strep infections.

One hallmark of rheumatic fever is the presence of antibodies that react with muscle tissue, particularly heart tissue, in a patient's serum. See "Rheumatic Fever" by Earl H. Freimer and Maclyn McCarty; Scientific American, December 1965. Normally, antibodies are not made against one's own tissues. Researchers have discovered, however, that so-called cross-reacting antibodies can sometimes be induced by a molecule in an infective organism that resembles one in the mammalian host. In the process of making antibodies against the microbial molecules to clear an infection, the body is tricked into generating antibodies against its own tissues (serological cross-reactivity), a potentially harmful development.

It is evident from this description that there is an important and urgent need for a vaccine which is effective against the various serotypes of group A streptococci. The vaccine should be capable of raising sero-specific antibodies, especially those capable of triggering acute rheumatic fever, without eliciting cross-reaction with human tissue. There is also an important need for a vaccine which has not only these properties but also is capable of raising protective antibodies against infections, sore throat, skin infections, deep tissue infections and the like that are not necessarily but frequently are followed by rheumatic fever. The invention contributes to solving these important needs in human health.

Thus, there is an important need for a vaccine effective against streptococci infections which provides humoral immune against the diverse serotypes of group A streptococci and, when desired, also cellular immune responses. The vaccine should not elicit antibodies which react with human heart tissue.

In conjunction with studies of the M protein of various serotypes, it has been found that in most cases the protective epitopes of M protein may be separated from the potentially harmful,

autoimmune epitopes of the molecule (see Refs. 5-7). The NH_2-terminal segments of M proteins have been found to evoke antibodies with the greatest bactericidal activity.

Further studies have shown that synthetic peptides copying limited regions of types 5, 6 and 24 M proteins evoked type-specific, opsonic antibodies that were not heart tissue cross-reactive. Because of their lack of immunogenicity, however, it was necessary to chemically link the synthetic peptides covalently to carrier proteins (see Refs. 5-7). Such fragments of M proteins linked to carrier proteins with chemical reagents do not result in hybrid proteins of defined structures. Thus, it has not been possible to obtain antigens which can elicit specific, desired antibodies without causing an increase of the risk of undesirable side reactions. Further, formation of hapten - carrier complexes using chemical cross-linking reagents is time-consuming and costly and results in undefined heterogenous mixtures of vaccine components. This invention provides multivalent vaccines that are type-specific and do not have the drawbacks of the prior art.

SUMMARY OF THE INVENTION

This patent application is related to and is co-filed on the same day as patent application No. entitled "ANTIGEN OF HYBRID M PROTEIN AND CARRIER FOR GROUP A STREPTOCOCCAL VACCINE" with named inventor James B. Dale (attorney's docket no. 372.5770P).

The invention relates to a recombinant multivalent hybrid M protein vaccine against multiple serotypes of group A streptococci. The vaccine comprises a protein molecule which contains epitopes (antigenic determinants) that evoke opsonic antibodies against multiple serotypes of group A streptococci. The molecule contains one amino acid fragment which contains at least one epitope which evokes humoral, opsonic antibodies of a particularly serotype. The respective fragments are optionally linked in tandem by linkers which include amino acids. The M protein is free of epitopes

that raise antibodies cross-reactive with the human heart tissue. The hybrid M protein is made by recombinant DNA technology.

The multivalent hybrid M protein contains amino acid fragments which are amino terminal fragments of the M protein and hence are capable of eliciting the desired opsonic antibodies against the multiple serotypes of group A streptococci. The invention also relates to multivalent hybrid M proteins which, in addition to amino acid fragments which evoke opsonic antibodies against specific serotypes of group A streptococcus, also carry amino acid fragments with epitopes which also evoke protective, cellular or mucosal antibodies.

In the hybrid M protein of the invention, the amino acid fragments need not all be immunogenic as such, yet when co-expressed with other amino acid fragments, the hybrid molecule is immunogenic against at least more than one serotype of group A streptococci.

Of particular interest are recombinant multivalent hybrid immunogenic M proteins which comprise amino terminal fragments of the M protein which contain epitopes that elicit antibodies which are causative of rheumatic fever, such as serotypes 1, 3, 5, 6, 14, 18, 18, 24, 27 and 29 or others. Ideally, the hybrid M protein is constituted of a multiplicity of amino acid fragments preselected to elicit antibodies against the particular target serotypes of group A streptococci.

The invention also relates to a vaccine of the M protein type which is multivalent or general, such as tetra, penta or hexavalent against the corresponding number of serotypes of group A streptococci. Thus, the invention relates to hybrid proteins which comprise different amino acid fragments of the C-repeats of an M protein of different serotypes which cause cellular immune responses, particularly those which are not tissue cross-reactive. Such hybrid proteins can also include fragments of the amino-terminal of the various serotypes to give a hybrid composite which elicits a humoral immune response and one or more cellular immune responses. The multivalent vaccines may contain one or more than one, i.e. repeats of any particular amino terminal fragment of different serotype.

Further, the fragments need not, but may be of the same amino acid lengths. Further, the invention contemplates a mixture or a "cocktail" of hybrid M protein which carry appropriate epitopes to elicit the desired opsonic antibodies against the target serotypes. The mixture of these hybrids will be effective against a large number, if not all, of the target streptococcal types, particularly those which are causative of rheumatic fever.

The invention also relates to mixtures of individual hybrid M protein molecules which are capable of not only eliciting opsonic antibodies against multiple serotypes of group A streptococci but also to elicit mucosal antibodies. In this manner, the invention provides as desired, a complete vaccine not only against those serotypes more likely to initiate or cause rheumatic fever, but also those which cause infections described above, which often can lead to rheumatic fever.

In accordance with the invention, the amino acid fragments which carry the desired protective epitopes (opsonic or mucosal) may be (but need not be) fused to each other in tandem by linkers which comprise amino acids. A great variety of such amino acid linkers can be used in accordance with the invention. It is desirable that the amino acids contribute to the orientation, conformation and in effect to the immunoaccessability of the epitopes of the fragments so as to generate an optimum immune response. It is not excluded that these amino acid linkers contain one or more molecules which are not amino acids.

In accordance with the invention, the order, i.e., the sequence of the amino acid fragments that constitute the hybrid M proteins which carry the protective epitopes can be changed as desired to maximize the immunogenicity of the molecule.

As referred to above, the invention relates to hybrid M proteins which include amino acids of the amino termini of the M proteins, i.e. M24, M5, M6 and M19 and amino acids of the carboxyl-terminus of type 5M protein. This vaccine raises type-specific opsonic antibodies against all of the related M fractions, cross-protective mucosal immune responses against two or more of these and cellular immunity.

The invention also relates to a particular PCR method which permits to organize the coding nucleotide sequences to express the desired amino acid fragments (or sequences) in the order desired.

The invention also relates to a method of immunization with the therapeutic recombinant multivalent hybrid M proteins of the invention or with a composition which comprises the recombinant hybrid multivalent M protein and an appropriate biochemically or pharmaceutically-acceptable carrier. The immunogenic hybrid M proteins of the invention may be formulated with the biochemically or pharmaceutically-acceptable carrier to produce a vaccine which elicits an effective level of the desired antibody in the subject mammal, including human beings, to provide the desired immunity, i.e. humoral or humoral and cellular.

Further, the invention relates to avirulent microorganisms transformed (or transfected) with recombinant multivalent hybrid M protein genes (or portions) thereof of the invention. The microorganism can be avirulent as such or may have been rendered non-virulent by methods known in the art. The avirulent host bacterium is unable to colonize in the subject to be immunized generally by virtue of a nutritional deficiency; nonetheless the bacterium will multiply just to a limited extent to release the M protein antigen and elicit the appropriate antibodies. Such compositions are very well suited for oral administration.

The invention also relates to the hybrid genes which code for and express the desired hybrid M proteins in an appropriate self- replicative vehicle.

The invention further provides for modification of the amino acid sequences constituting the hybrid antigenic molecule by chemical method if it is desired to add to and/or replace any one of the amino acids by another molecule to increase and/or modify the immunogenicity of the hybrid molecule.

This summary of the invention is not intended to summarize all the embodiments (or aspects) of the invention.

BRIEF DESCRIPTION OF THE FIGURES

FIG.1 shows the recombinant DNA nucleotide sequence of recombinant and trivalent amino acid sequences of M24-M5-M6.

FIG. 2 shows the immunoblot analysis of purified M24-M5-M6.

FIG. 3 shows ELISA inhibition assays.

FIG. 4 shows the recombinant DNA nucleotide sequence and deduced amino acid sequence of tetravalent amino acid sequence of tetravalent M24-M5-M6-M19 hybrid molecule.

FIG. 5 shows the immunoblot analysis of M24-M5-M6-M19 hybrid vaccine.

FIG. 6 shows the DNA recombinant nucleotide sequence and deduced amino acid sequence of tetravalent hybrid M24-M5-M6-M19 with different linkers.

FIG. 7 shows the DNA recombinant nucleotide sequence and deduced amino acid sequence of tetravalent hybrid M24-M5-M6-M19 constituted of repeats of smaller fragments of each of the different serotypes with these fragments being directly linked by their respective amino and carboxy ends to the adjoining fragment at the indicated restriction sites.

FIG. 8 shows the DNA recombinant nucleotide sequence of tetravalent hybrid M24-M5-M6-M19-C-terminus of 915 nucleotides encoding the 305 amino acid long hybrid by having the COOH terminal half of M5 joined at restriction site Pst1.

FIG. 9 shows the DNA recombinant nucleotide sequence and deduced amino acid sequence of the tetravalent hybrid M19-M6-M5-M24 where the sub-units are in the reverse order than in construct shown in FIG. 4. No linkers join the fragments of the nucleotide and the of the amino acids.

FIG. 10 shows the DNA recombinant nucleotide sequence of a divalent M24-M5 hybrid.

FIG. 11 shows the DNA recombinant nucleotide sequence of tetravalent M19-M6-M5-M24 of 1029 nucleotides long linked to C-repeats of the carboxy terminal.

FIG. 12 shows the DNA recombinant nucleotide sequence of tetravalent M24-M5-M6-M19 with short 15 amino acid units linked directly to each other.

FIG. 13 shows the DNA recombinant nucleotide sequence of octavalent hybrid protein M24-M5-M6-M19-M4-M1-M18-M12 which contains a non-rheumatogenic serotype of streptococci (M12).

Suitable vectors for cloning the selected DNA fragments of the M protein are available commercially. See the Bibliography provided herewith. Expression of the hybrid protein is carried out by suitable prokaryotes, like E. coli or if desired eukaryotes, like yeast.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Various embodiments of the invention are more fully described hereinafter.

The construction of a trivalent and of a tetravalent hybrid antigen is described in greater detail, but it is understood that similar protocols are applicable to construct the other hybrid antigens described herein.

The method of the invention for constructing the multivalent hybrid constructs involves in vitro recombinant DNA technology. The method may be described in a general manner as follows. A polyvalent hybrid gene is constructed using a selected native fragment of the desired length and is constituted of a desired M gene like of M24, 1, 5, etc. (designated emm24, emm1 and emm5, respectively). The fragment is free of nucleotides that encode an amino acid sequence that can cause tissue cross-reactivity. The DNA sequences encoding amino acid fragments free of epitopes which cause autoimmune responses are identified as shown in the literature, for instance References 5, 6, 7 in the attached Bibliography. The fragment is amplified by polymerase chain reaction (PCR), purified and ligated into a self-replicating vehicle, e.g., a plasmid, like pBR322. Oglionucleotide pairs copying the desired codons of the structural other genes that make up the multivalent gene, respectively, are synthesized to contain appropriate restriction sites to facilitate ligation to the first

selected gene, e.g. emm24. The entire polyvalent construct is excised from the vehicle (e.g., pBR322) and ligated to allow high level expression of the recombinant hybrid protein in an appropriate plasmid e.g. pKK223-3.

The selected gene (e.g. emm24) is amplified by PCR using synthetic oglionucleotide primers that specify amplification of the gene that encodes the desired region of the amino-terminal portion e.g., half of the molecule, e.g., the pep M24 region following the procedures and materials disclosed in Beachey et al., 1978 entitled "Repeating Covalent Structure of Streptococcal M Protein, Proc. Natl. Acad. Sci. USA, 75, 3163-3167 and Mouw et al., 1988, entitled "Molecular Evolution of Streptococcal M Protein, J. Bacteriol., 170, 676. With respect to the structural gene of type 24M, the complete nucleotide sequence includes an open reading frame of 1617 base pairs encoding a pre-M24 protein of 539 amino acids a predicted M.W., of 58,738. The structural gene contains two distinct tandemly reiterated elements. The first repeated element consists of 5.3 units, and the second contains 2.7 units. Each element shows little variation of the basic 35-amino acid unit.

The (top and bottom PCR) primers are synthesized by an automated DNA synthesizer (ABI, Model 381A). When necessary i.e. when the region to be amplified does not contain an appropriate signal sequence and native start codon, the top strand primer is extended on the 5' end by an appropriate start codon like ATG.

This is performed in the case of emm24. Appropriate restriction sites (e.g. EcoR1) are also added and a GGG tail incorporated to ensure that the enzyme would recognize the cleavage site in the purified, double stranded DNA product. The bottom strand primer is extended on the 5' end by an appropriate restriction site (e.g., BamH1) and a CTC tail.

PCR amplification is performed using chromosomal DNA extracted from the selected type streptococci (e.g. type 24), which is then used as template. See Dale et al., Type-Specific Immunogenicity of a Chemically Synthesized Peptide Fragment of Type 5 Streptococcal M Protein, J. Exp. Med., 158, 1727 (1983). The reaction mixtures comprise template DNA, primer pairs, dNTPs and Taq DNA polymerase in PCR buffer. Amplification is performed in an appropriate automatic

thermal cycler with denaturation, primer annealing and primer extension in accordance with standard methods. The PCR product is electrophoresed by known methodology and the purified product is, if necessary, repaired using Klenow fragment and cut sequentially with the appropriate restriction enzymes, in this case EcoR1 and BamH1 in appropriate buffers.

The purified PCR product (e.g. emm24) is ligated into an appropriate self-replicating vehicle such as pBR322 cut with restriction enzymes like EcoR1 and BamH1. The plasmid is used to transform E. coli strain mC1061 by standard method. Transformants are screened for the presence of plasmids containing inserts of the appropriate size and gels and one plasmid for purification and ligation of the other synthetic oglionucleotide pairs previously synthesized.

The selected oglionucleotides (emm5) are mixed in equi-molar ratios and allowed to re-anneal. The selected oglionucleotide pair (e.g. emm5) is then ligated to the purified cut plasmid used to transform an appropriate strain of E. coli, such as mC1061. Plasmids containing appropriately sized inserts are identified and one is selected for ligation of the additional oglionucleotide pairs (e.g. emm6) which is accomplished in a similar manner. The resulting polyvalent plasmid e.g. pCDM24 M5-M6 is then purified and cut with restriction enzymes. The excised polyvalent hybrid gene (e.g. emm24-5-6) is then ligated into a high level expression vector (e.g. pKK223-3) that contains a promoter and a ribosome binding site adjacent to the cloning side, e.g. EcoR1.

In a similar manner, other multivalent hybrid gene constructs are synthesized. For instance the M24-M5-M6-M19 tetravalent hybrid protein is constructed using fragments of the 5' region of the emm genes amplified by PCR, purified, ligated and tandem and expressed in pKK223-3.

Sequence analysis of the polyvalent hybrid emm genes are confirmed by sequencing appropriate inserts in an appropriate vehicle, such as pKK223-3 by the dideoxy-nucleotide chain termination method of Sanger et al., DNA Sequencing with Chain-Terminating Inhibitors, Proc. Natl. Acad. Sci. USA, 74, 5463 (1977).

The method of the invention includes the purification of the recombinant hybrid M proteins. These are purified from extracts of an E. coli, cells are pelleted and treated by standard purification methods, and finally lyophilized.

The invention also includes a method for synthesizing by PCR a recombinant DNA sequence which is constituted by the desired nucleotide in the particular order (sequence) and of the particular size and orientation desired. Considering that the objective is to synthesize a DNA sequence constituted of sequences A-B-C-D the following procedure is followed. After cutting PCR-generated fragment A and B with the desired restriction enzyme at the unique sites encoded by 5' end of PCR primers, A and B are ligated to each other. The A-B fragment is amplified using as primer the A top strand oligonucleotide and the B bottom strand oligonucleotide, thus amplifying only the A-B ligation product. This product is ligated to fragment C. The A-B-C fragment is in turn PCR amplified using the top oligonucleotide strand of A as primer and the C bottom strand oligonucleotide as primer, thus only amplifying the A-B-C strand.

This product is ligated to fragment D. The fragment A-B-C-D is in turn amplified using the top oglionucleotide strand of A as primer, and the bottom strand of D as primer, thus amplifying only the A-B-C-D strand desired.

In a variant of the procedure, A-B fragment can be amplified, C-D synthesized, as described for A-B. Then A-B and C-D are ligated and PCR amplified using the top oglionucleotide strand of A as primer and the D bottom strand oligonucleotide, thus amplifying the desired A-B-C-D sequence.

If it is desired to incorporate additional sequences containing for instance, amino acids as linkers (as described herein) between the amino acid segments, these molecules (e.g. amino acids) can be encoded by nucleotides contained in the primers utilized to amplify the desired fragment(s).

Thus, the PCR-generated fragments are ligated in the desired order and portions are PCR amplified using the upper and lower strands as illustrated. The method avoids arrangements of

nucleotide segments in an undesired sequence or orientation. The same methodology is followed for longer fragments and variations can be readily provided.

The polyvalent hybrid M proteins of the invention are tested by immunization of rabbits, a classic test animal. Assay for M protein antibodies is performed in accordance with known methods as described in the appended bibliography. So are assays for heart cross-reactive antibodies which are described in Beachey and Dale, (1982).

The invention also includes the construction of hybrid constructs containing repeating amino-terminal M protein sub-units using PCR. A non-limiting illustration is described hereinafter. The method is readily applied to any number of amino acids of any particular selected serotype to yield a hybrid gene containing its selected repeated amino acid fragment.

A typical trivalent gene M24-M5-M6, was constructed in a general manner as follows.

The M24-M5-M6 hybrid gene was constructed using a native fragment of the emm24 gene that was amplified by the polymerase chain reaction (PCR), purified and ligated into pBR322. Oligonucleotide pairs copying the first 11 and 12 codons of the structural emm5 and emm6 genes, respectively, were synthesized to contain appropriate restriction sites to facilitate ligation to the emm24 gene. The entire trivalent construct was excised from pBR322 and ligated into pKK223-3 to allow high-level expression of the recombinant hybrid protein.

The emm24 gene was amplified by PCR using synthetic oligonucleotide primers that specified amplification of the portion of the gene that encodes the pep M24 region of the protein which is approximately the amino-terminal half of the molecule. The primers were synthesized by an automated DNA synthesizer (ABI, Model 381A) and had the following structures:

M24-Top Strand PCR Primer

-EcoR1 START

5' GG GAA TTC ATG GTC GCG ACT AGG TCT CAG 3'

M24-Bottom Strand PCR Primer

5' CGT CTC TTT CGA CTA GAA CTT CCT AGG CTC 5'

-BamH1-

The top strand primer was extended on the 5' end by an ATG, since the region of emm24 to be amplified excluded the signal sequence and native start codon. An EcoR1 restriction enzyme site was also added and a GGG tail was incorporated to ensure that the enzyme would recognize the cleavage site in the purified, double-stranded DNA product. The bottom strand primer was extended on the 5' end by a BamH1 restriction site and a CTC tail.

PCR amplification was performed using chromosomal DNA extracted from type 24 streptococci which was used as the template. The reaction mixtures consisted of template DNA, primer pairs, dNTPs and Taq DNA polymerase in PCR buffer. Amplification was performed in a Perkin-Elmer Cetus automatic thermal cycler with denaturation at 95^0C for 1 min, primer annealing at 55^0C for 1 min and primer extension at 72^0C for 3 min for a total of 39 cycles. The PCR product was electrophoresed in a 1% low melting-point agarose gel, the band of the predicted size was excised and purified by adsorption to and elution from "glassmilk" (Geneclean, Bio 101). The purified product was end-repaired using Klenow fragment and cut sequentially with EcoR1 and BamH1 restriction enzymes in the appropriate buffers.

The purified emm24 PCR product was ligated into pBR322 that had been cut with EcoR1 and BamH1. The plasmid was used to transform E. coli strain MC1061 by standard methods. Transformants were screened for the presence of plasmids containing inserts of the appropriate size on agarose gels. One such plasmid (pCDM24) was then selected for purification and ligation of emm5 and emm6 synthetic oligonucleotide pairs which were synthesized as described above according to the following sequences:

emm5 synthetic oligonucleotide pairs

1/2 BamH1 1/2 Sal1

5' GA TCC GCC GTG ACT AGG GGT ACA ATA AAT GAC CCG CAA G 3'

3' G CGG CAC TGA TCC CCA TGT TAT TTA CTG GGC GTT CAG CT 5'

emm6 synthetic oligonucleotide pairs

1/2 Sal1 -pst 1- 1/2 XbaIII

5' TC GAC AGA GTG TTT CCT AGG GGG ACG GTA GAA AAX XXG GAC CTG CAG 3'

3' G TCT CAC AAA GGA TCC CCC TGC CAT CTT TTG GGC CTG GAC GTC GCC GG 5'

The emm5 oligonucleotides were mixed in equimolar ratios, heated at 65^{0}C for 2 min and allowed to reanneal at ambient temperature. pCCDM24 was cut with BamH1 and Sal1 and purified on an agarose gel as described above. The emm5 oligonucleotide pair was then ligated to the purified, cut plasmid and used to transform E. coli strain MC1061. Plasmids containing appropriate sized inserts were identified on agarose gels and one (pCDM24.M5) was selected for ligation of the emm6 oligonucleotide pairs which was accomplished in a similar manner. The resulting pCDM24.M5-.M6 was then purified and cut with EcoR1 and Pst1, the site for which was synthesized into the emm6 oligonucleotide pair. The excised emm24.5.6 hybrid gene was then ligated into pKK223-3, a high level expression vector that contains the tac promotor and a ribosome binding site adjacent to the EcoR1 cloning site.

The construction, cloning and expression of M24-M5-M6-M19 tetravalent hybrid M protein was carried out as follows.

The M24-M5-M6-M19 tetravalent M protein was constructed using fragments of the 5' regions of emm genes that were amplified by PCR, purified, ligated in tandem and expressed in pKK223-3. The overall goal was to amplify the regions of the respective emm genes that encode protective and not tissue-cross-reactive epitopes and link them into one protein molecule. The recombinant hybrid protein contained 113 amino-terminal amino acids of M24, 58 amino acids of M5, 35 from M6 and 35 from M19. Each segment was linked by 2 amino acids specified by the respective

restriction enzyme sites that were synthesized into the oligonucleotide primers used to specify the PCR products.

The primers for each emm gene were synthesized as described above according to the following sequences:

M24 Top Strand

-EcoR1- Start

5' GG GAA TTC ATG GTC GCG ACT AGG TCT CAG 3'

M24 Bottom Strand

-BamH1-

5' GG GGA TCC TTC AAG ATC AGC TTT CTC TGC 3'

M5 Top Strand

-BamH1-

5' GGG GGG GGA TCC GCC GTG ACT AGG GGT ACA 3'

M5 Bottom Strand

-Sal1-

5' GGG GGG GTC GAC CTC AGT TTT TAA CCC TTC 3'

M6 Top Strand

-Sal1-

5' GGG GGG GTC GAC AGA GTG TTT CCT AGG GGG 3'

M6 Bottom Strand

-Nco1-

5' GGG GGG CCA TGG TAA GTT GTC AAT AAT AGC 3'

M19 Top Strand

-Nco1-

5' GGG GGG CCA TGG AGA GTG CGT TAT ACT AGG 3'

M19 Bottom Strand

-Pst1-

5' GGG GGG CTG CAG AGA TAA CTT CTC ATT CTG 3'

The M24, M5 and M6 oligonucleotide sequences were based on previously published data. See Bibliography supplied herewith and incorporated herein by reference. The M19 sequence was obtained in a similar manner from a plasmid containing the entire emm19 structural gene.

The oligonucleotide primer pairs described above were used to amplify the regions of each emm gene using chromosomal DNA from the respective serotype of group A streptococci as the template in the PCR reaction, as described above. The PCR products were purified by excision from low melting point agarose. Because some of the bottom strand PCR primers annealed to regions of the emm genes that were repeated, the PCR products were of variable sizes. In each case, the smallest major band was excised and purified for ligation.

Ligation of the purified PCR products was accomplished by first cutting the M24 and M5 fragments with BamH1 and then ligating the two cut fragments. The ligation mixture was then subjected to amplification by PCR using as primers the M24 top strand oligonucleotide and the M5 bottom strand oligonucleotide in order to amplify only the M24-M5 ligation product. This hybrid PCR product were then purified, cut with Sal1 and ligated to the M6 fragment that had also been digested with Sal1. The M24-M5-M6 hybrid was once again subjected to PCR amplification using the M24 top strand primer and in this case the M6 bottom strand primer. The same sequence of events was then followed to ligate the M19 component to make the completed tetravalent gene. The purified products was cut with EcoR1 and Pst1 and ligated into the respective sites of pKK223-3 which was used to transform E. coli strain JM105. The recombinant expressing the hybrid M protein was initially identified by screening colony blots on nitrocellulose with rabbit antisera against pep M24.

The constructing of repeating amino-terminal M protein sub-units using PCR was carried out as follows.

Multimeric amino-terminal fragments of emm genes were constructed using PCR amplification. For example, the first 12 amino acids of M19 were expressed as three repeats by the following method:

PCR Primers:

M19-5' repeater - 5' AGA CTG CGT TAT ACT AGG CAT ACG CCA GAA GAT AAG AGA GTG CGT TAT ACT AGG

M19-3' BS - 5' GGG GGG CCA TGG CTT ATC TTC TGG CGT ATG

M19-5' Monomer - 5' GGG GGG GAA TCC AGA GTC CGT TAT ACT AGG

The M19-5' repeater encodes the first 12 amino acids of the NH_2 terminus with the codons for the first 6 amino acids added to the 3' end of the primer as a repeating sub-unit. M19-3' BS copies the complementary strand of codons 7-12 with an Nco1 enzyme site and a poly-G clamp. The M19-5' monomer encodes the first 6 amino acids of the NH_2 terminus with an EcoR1 enzyme site and a poly-G clamp at the 5' end.

Template DNA from type 19 streptococci was first amplified with the M19-5' repeater and M19-3' BS which resulted in a ladder of PCR products ranging in size from 2 to ~6 sub-units. One product of the appropriate size (to encode 3 sub-units) was purified from agarose gels and then subjected to PCR amplification using the M19-5' monomer and M19-3' BS primers. The single product was then ligated into the appropriate restriction sites.

The sequence analysis of the trivalent and tetravalent hybrid emm genes was carried out as follows.

The structures of the hybrid emm genes described above were confirmed by sequencing the inserts in pKK223-3 by the dideoxy-nucleotide chain termination method of Sanger et al..

The purification of recombinant hybrid M proteins was carried out as follows. The trivalent and tetravalent hybrid M proteins were purified from extracts of JM105 E. coli grown overnight in 11 L broth supplemented with 75 ug/ml ampicillin, 25 ug/ml streptomycin and IPTG

(1mMol). The cells were pelleted at 7000 xg and resuspended in 50 ml carbonate buffer, pH 11.0 containing 100 ug/ml lysozyme, 1 mMol EDTA and 100 ug/ml PMSF and incubated at 37^{0}C for 30 min. The cells were centrifuged at 7000 xg and the supernatant was dialyzed against distilled water and lyophilized. Purification was performed by loading 50 mg of extract containing either the trivalent M protein hybrid onto a preparative PAGE unit (Prep Cell, Model 491, Bio Rad., Inc.) using a 37 mm column and a 9 cm 11% polyacrylamide gel. Six ml fractions were collected and assayed for the presence of recombinant proteins by Western blot analysis using pep M24 antisera. Peaks containing activity were polled and lyophilized.

Immunization of Rabbits

Rabbits were immunized with 300 ug of the selected polyvalent (e.g. the trivalent or tetravalent) hybrid M proteins emulsified in complete Freund's adjuvant. Booster injections of the same dose in PBS were given at 4, 8 and 12 weeks. Blood was obtained prior to immunization and at two-week intervals thereafter. The other polyvalent hybrid M proteins are used in the same manner. Likewise, a mixture (or "cocktail") of such hybrids are used in the same manner.

Assays for M Protein Antibodies

Total antibody activity against M protein was determined by ELISA using native pep M proteins, recombinant M proteins, synthetic peptides or purified polyvalent (e.g. trivalent or tetravalent) hybrid M proteins as solid phase antigens by methods previously described. Opsonic antibodies were assayed by in vitro opsonophagocytosis tests, as described. ELISA-inhibition and opsonization-inhibition experiments were performed using purified M proteins or synthetic peptides as soluble inhibitors of M protein antibodies in ELISA or opsonization tests. Western blots of recombinant proteins were performed using antisera raised in rabbits against synthetic peptides or native M proteins. The other polyvalent hybrids were assayed following the same protocol.

Assays for Heart-Cross-Reactive Antibodies

Antisera against recombinant multivalent M proteins were screened for the presence of heart-cross-reactive antibodies by indirect immunofluorescence tests using frozen sections of human myocardium as previously described in the literature.

Assays for M Protein Epitopes that Evoke Mucosal Antibodies

Broadly Protective Against Infection

Rabbit antisera were screened for the presence of broadly protective antibodies using passive mouse protection assays (see Bronze, M.S., et al., Protective and Heat-Crossreactive Epitopes Located within the N-Terminus of Type 19 Streptococcal M Protein, J. Exp. Med., 167, 1849-1859 (1988). Antisera were first tested for the ability to react with the surface M protein of multiple heterologous serotypes of group A streptococci by ELISA. Those that recognized M protein epitopes in their native conformations were then used to passively protect mice against intranasal challenge infections. Antibodies were absorbed to virulent streptococci and mice were challenged intranasally with 10^7 CFU. Throat cultures were obtained on alternate days and deaths were counted over the ensuing 14 days. Vaccine constructs that evoke protective antibodies in rabbits will be used to immunize mice intranasally to test directly their protective immunogenicity. Actively immunized mice will be similarly challenged with virulent streptococci.

An illustration of a tetravalent hybrid gene M24-M5-M6-M19 with different linkers than shown in the FIG. 4, is shown in FIG. 6. The sequence of the hybrid gene shows the structure of emm24, emm5, emm6 and emm19. The tetravalent emm gene expresses protein with a calculated M.W. of 30.7 kDA and contains 113-amino terminal amino acids of type 24 M protein, 58 amino acids of type M5, protein 35 amino acids of type 6M protein, and 35 amino acids of type 19 M protein. The linker is a proline rich linker Pro-Gly-Asn-Pro-Ala-Val-Pro, the codons for which are inserted into the BamH1, Sall and Nco1 restriction enzyme sites, respectively, which were

synthesized into the original PCR primers. This linker in part includes the amino acids of the restriction site codon at the desired position.

Other linkers may be used such as for instance a sequence which include amino acids like, Ile-Pro-Gly or Asp-Pro-Arg-Val-Pro-Ser-Ser.

The sequence of the amino acid in any particular linker used appears at this time not to be critical. Theoretically, a linker could be constituted by one amino acid; if the desired effect of promoting a functionally effective conformation of the encoded protein is desired, longer linkers may be selected, such as of having 14 or more (e.g. 20) amino acids.

As described herein, linkers are not essential to the structure so that it is not necessary that any one amino acid sub-unit or for that matter all amino acid by fused to each other by an amino acid linker. For an illustration, M24 and M5 can be directly fused to each other. Further, while the description herein refers to linkers constituted by amino acids encoded by hybrid genes, some hybrid constructs may contain purified recombinant M proteins which may include one or more molecules which are not an amino acid, such as succinimidyl-4-(N-maleimido-methyl) cyclohexane-1-carboxylate (SMCC). The linkers can be of same or of varying lengths between each amino acid segment. These molecules may be introduced by chemical means as opposed to being expressed with the hybrid protein.

The tetravalent hybrid gene illustrated in FIG. 6, when tested for antigenicity by reacting with polyclonal rabbit antisera raised against each one of the components of the hybrid protein, will indicate that the epitopes are present in a conformation which resembles that of the native protein.

When the immunogenicity of the tetravalent protein and the antibody level will be determined, the immune sera will also contain opsonic antibodies against all four serotypes of group A streptococci.

It is contemplated in accordance with the invention that the amino terminal amino acid fragment be constituted to contain one or more amino-terminal portions of other potentially

rheumatogenic streptococci types, for instance of types 1, 3, 18, 27 and/or 29 or any other presently known or to be discovered to have such potential rheumatogenic effect. Also it is contemplated that the constructs of the invention be constructed to contain one or more fragments of the amino terminal region of serotypes which are not known to have such rheumatogenic effect, as those described above and in the literature. In those instances where such structure have not yet been sequenced or when such sequence has not yet been published, one skilled in the art by methods readily available can sequence such structures and then construct the hybrid of the invention with the desired fractions. Thus, the invention contemplates such multivalent protein encoded by appropriate hybrid gene or genes to express in an appropriate organism a protein that will elicit the desired antibodies.

The effect of the different linkers on the immuogenicity of the hybrid molecule may justify further investigations. It is not excluded that depending on the nature of the linker and of the type and size of the amino acid fractions, a hybrid protein of ideal or close to ideal high immunogenicity be identified. Such hybrid is within the scope of the invention.

What has been described herein above also applies with respect to the carboxy-terminal fraction or the C-repeats thereof when such fraction(s) or repeats are used, as described herein.

It should be kept in mind that not all - instead none - of the fragments constituting the hybrid need be immunogenic when considered individually (and without a carrier) provided that when part of the final hybrid they contribute to the desired immunogenicity or at least do not detract therefrom.

The sequence of amplified (M-like) 2, 3, 18 and 19 M genes is discussed in Podbielski et al., Application of the Polymerase Chain Reaction to Study the M Protein(-like) Gene Family in Beta Hemolytic Streptococci, Med. Microbiol. Immunol., 180, 213 (1991). Genes of the M12 type (emm12) of a nucleotide sequence of 1693 base pairs is described in Robbins et al., Streptococcus Pyogenes Type 12 Protein Gene Regulation by Upstream Sequences, Journal of Bacteriology, 5633-5640 (Dec. 1987). The NH_2-terminal sequence of type 1 streptococcal M protein is discussed in Kraus et al., Sequence and Type-Specific Immunogenicity of the Amino-Terminal Region of Type 1

Streptococcal M Protein, The Journal of Immunology, 139, 3084-3090 (Nov. 1987), incorporated by reference.

The NH_2-terminal fragment is constituted of fragments of 28-kDA, 25-, and 23.5 kDA. The article discusses similarities and differences with other NH_2-terminal M protein sequences. Opsonic antibodies are developed against type 1 streptococci. It is noteworthy that the NH_2-terminal region of type 1M protein also retains epitopes that evoke protective immune responses.

It is therefore within the scope of the invention for the hybrid construct to contain NH_2-terminal regions which also raise protective mucosal responses, (not only opsonic responses) in those instances where the NH_2-terminal region does raise both types. Thus, the carboxy terminal fragment is not always necessary for a hybrid to raise mucosal or cellular responses.

In general, the NH_2-terminal residues of the different M proteins which show less ordered structure and are more variable from one type to another comprises about 10 to 20 residues.

Another embodiment of the invention is illustrated in Figure 7 which shows a M24-M24-M24-M5-M5-M5-M6-M6-M6-M19-M19-M19 multivalent hybrid of 561 nucleotides and a calculated M.W. of 21.6 kDA constituted of 187 amino acids with restriction sites between the different fragments as shown, respectively BamH1, Sal 1, and Nco1. It will be observed that the repeated amino acid fractions of the respective types M24, M5, M6 and M19 (underscored) are of the same length. They need not be so. The smaller size of the repeat fragments of the construct is to enhance the immunogenicity of the entire molecule as opposed to longer fragments as described elsewhere herein and to evoke antibodies against the distal (and most protective and least tissue-cross-reactive) epitopes. Each fifteen amino acid sub-unit is repeated three times. Further, such smaller amino acid fragments can be more readily synthesized by an amino acid synthesizer or by a novel modification of the classic PCR method as described herein. In this embodiment of the invention, it will observed that there are no linkers.

Contemplated within the invention are similar hybrid structures in which the individual repeated segments are longer or shorter than the shown 15 amino acids. Since the

individual M fragments constituting the hybrid are when considered alone, of different immunogenicity, it appears worthwhile to consider increasing or decreasing the length of one or more of such fragments to further increase the overall immunogenicity of the molecule. Linkers can also be included.

Further, anyone of the fragments here illustrated in FIG. 1 hereinabove can be replaced by another serotype, such as serotype 1, 3 or 18. In this manner, tetravalent hybrid genes can be constructed and the corresponding hybrid protein expressed. Likewise longer, such as penta-, hexa-, octa-, nona- or decavalent hybrid genes and the corresponding expressed proteins can be obtained.

In that connection, it is noteworthy that as opposed to increasing the number of amino acid fragments constituting a particular multivalent vaccine, such as to octa-, nona- or deca-multivalent vaccine, it would be more advantageous to provide a mixture of smaller constructs, the mixture being constituted by at least 2 of such constructs. In this manner, it may be a mixture of "cocktail" of multivalent vaccines can be provided which would have an optimum maximum length (or size) such as tetra- or pentavalent structure and another one of approximate similar length and constituted of other serotypes. For instance, a multivalent hybrid vaccine constituted of M24-M5-M6-M19 can be provided in admixture with one containing M1-M3-M18 and a further admixture with a multivalent hybrid vaccine constituted of M1-M24-M5 and yet a fourth one containing any one of those above mentioned including M18. It is to be observed that the order (sequence) in which these M protein fragments have been described herein is not necessarily the sequence in which the invention is limited as has been described repeatedly herein.

Another interesting illustration of a tetravalent hybrid gene of the invention is illustrated in Figure 8 which shows the sequence of tetravalent M24-M5-M6-M19 with the carboxy terminal half of M5 joined at restriction Pst1. The hybrid construct has 305 amino acids expressed by the 915 nucleotides. The restriction sites are shown.

It will be observed that the tetravalent amino acid fragments are each of 15 amino acids long and joined directly to each other without the intermediary of amino acid linkers. This is an illustration of the concept of the invention combining the multivalent vaccine with that of a carboxy terminal of one of the M protein serotypes.

Instead of using the carboxy terminal of M5 any other M-COOH may be used such as that of M24, M19 and M6. Care will be taken of course that the carboxy terminal not be one that would generate undesirable antibodies such as tissue-cross-reactive antibodies. In the illustrated construct, not only are there generated opsonic antibodies against the four M protein fractions, but also protective mucosal antibodies against the carboxy terminal portion of the molecule. As explained herein, such a structure has distinct advantages in that in can serve as a vaccine in controlling nasal or other infections often preceding rheumatic fever.

A vaccine constituted of the construct illustrated herein, or a similar one is therefore an effective, therapeutic prophylactic agent which interesting enough may be administered nasally as by spray.

Instead of using the entire carboxyl terminal of anyone of the M-serotypes, it may be advantageous to use only one or more amino acid of the C repeats of the carboxyl terminal of a particular serotype. It is noteworthy that the carboxy terminal or the amino acid constituting one or more C-repeats used in the construct need not be one of the same serotype(s) as that which constitutes the amino terminal portion of the construct. Thus, such vaccine will provide cellular immune responses (which normally are less type-specific or more cross-reactive) than the opsonic response and concurrently provide type-specific immunity.

It should be noted in conjunction with the invention as has been described herein, that not all M protein epitopes are sero-specific in their amino terminal portion of the molecule. Some epitopes of particular serotypes, such as M5 do cross-react to some extent with streptococci of a type other than M5, such as M6 or M19. And to some extent this also occurs with other M serotypes.

Accordingly, it is within the scope of this invention that when sero-specificity is referred to, this does not exclude some cross-reactivity between certain shared structures.

However, such shared epitopes are often also most likely to cross-react with heart tissue and hence present potentially serious risks and are not opsonic or do not evoke antibodies with a high level of opsonic activity desirable.

In another embodiment of the invention, the sequence of the amino terminal amino acids of the various fragments has been co-expressed in a different order. A comparison between on one hand, the structures of FIGS. 4 and 6 and on the other hand, FIG. 9 will show that the DNA recombinant nucleotide sequence and the deduced amino acid sequence of the tetravalent hybrid M19-M6-M5-M24 is in the reverse order than in the other above-mentioned constructs. The construct of Figure 9 illustrates a multivalent vaccine having 247 amino acids expressed from a nucleotide sequence of 741 DNA nucleotides. The respective M19, M6, M5 and M24 fragments are of the following respective amino acids length: 35, 35, 58 and 113.

In studies related to the invention, it had been found that the immunogenicity of certain sub-units or fragments is greater than others. For instance, M24 is greater than M5, which in turn is greater than M6 and which in turn is greater than M19. It was also observed that the immunogenicity against the amino terminal M24 and M5 sub-units in a construct containing M24-M5-M6-M19 was greater than against the M6 and M19 components. However, it was of interest in conjunction with this invention to determine whether such postulates would hold true in the constructs of the invention, in particularly, in a total reversal of the order of the amino acid sub-units as is shown in Figure 9 or for that matter, in the rearrangement of some of these sub-units in any order desired such as M19 followed by M6 and then followed by M24 and then by M5.

The same remarks apply with respect to the other M protein serotypes as 3, 12 and 18. Indeed it is not seen at this time why organizing the sequence of amino acids by increasing (or decreasing) immunogenicity should apply to the constructs of the invention. In that sense, the vaccines of the invention may present another interesting departure from the conventional.

The tetravalent protein reacted with polyclonal rabbit antisera raised against each component of the hybrid protein as described herein. Thus, indicating that the epitopes were present in a conformation which mimicked the native protein. Rabbits immunized with a purified tetravalent M protein are expected to develop significant antibody levels against the tetravalent vaccine of all four serotypes of the purified native M proteins. By changing the order of the amino acid fragments in the tetravalent hybrid, different levels of opsinization can be observed.

As described herein, when linkers are used, linkers which are of particularly interest are constituted to be overall hydrophobic i.e. they are constituted by a multiplicity of amino acids with non-polar groups. Included in such amino acids are those with aliphatic groups, such as alanine, leucine, isoleucine, valine and proline; with aromatic rings like phenyalaine and tryptophan, and methionine. It is in accordance with the invention that other of the 22 amino acids may be considered with the hydrophobic acids or not to form the appropriate linkages in those hybrid construct where linkages are desired.

An illustration of a divalent hybrid gene emm24 and emm5 and the amino acid sequence is shown in FIG. 10 of 522 nucleotides has expressing the hybrid protein of 174 amino acid. The restriction site BamH1 is shown.

Opsonic antibodies are elicited against the respective native M proteins. Immunogenicity is tested in rabbits.

An illustration of a tetravalent gene of the invention in FIG. 11 shows the sequence M19-M6-M5-M24 in the reverse order than shown in FIG. 8 with two and a half C-repeats of the M5-carboxyl terminal. The 1029 nucleotide long shows the restriction sites between the respective fragments and the fused C-repeats of a total of 280 nucleotides long of the carboxyl terminal amino acid region of M5.

This construct is noteworthy in that it will elicit not only antibodies against the M6-M19-M24-M5, but also against the C-repeats. Each complete C-repeat is 35 amino acids long, the

last one being approximately 1/2 thereof. The C repeats will generate mucosal protective antibodies. Thus, again this is an interesting multi-purpose vaccine.

In yet another embodiment of the invention, a multivalent hybrid M protein with short sub-units is illustrated in FIG. 12. FIG. 12 shows the DNA recombinant DNA nucleotide sequence and deduced amino acid sequence of tetravalent hybrid M24-M5-M6-M19 which comprises 201 nucleotides coding for 67 amino acids. Of interest in conjunction with this embodiment is the shortening of each sub-unit and linkers numbering 2 amino acids encoded by the shown restriction sites BamH1, Sal1 and Nco1. By shortening each sub-unit, the immunogenicity of each one can be determined. The total size of the molecule can be minimized. This allows for the construction of multivalent constructs to which other sub-units from heterologous serotypes of M proteins are co-fused. Thus, it can readily be seen that other rheumatogenic amino terminal fragments such as serotype M1-M3-M18 can be added to this construct to render the multivalent hybrid M protein to be of increased multivalency. Or, as described above, a mixture of such smaller structures may be provided as a vaccine to the patient.

When rabbits are immunized with the purified tetravalent M protein, significant antibody levels of all four serotypes of the purified native M protein is observable. The same construct can be made, omitting the short linkers.

In a further embodiment of the invention, there is illustrated in FIG. 13, an octavalent hybrid protein M24-M5-M6-M19-M3-M1-M18-M12. This octavalent hybrid protein vaccine is constituted of a fraction of M24 of 15 amino acids long, a fraction of M5 of 15 amino acids long, of a fraction of M6 of 15 amino acids long, a fraction of M19 of 15 amino acids long, a fraction of M3 of 15 amino acids long, a fraction of M1 of 15 amino acids long, a fraction of M18 of 15 amino acids long and a fraction of M12 of 15 amino acids long.

Each amino acid being connected by a 2 amino acid long linker to the following amino acid: the nucleotide sequence being 405 long and coding the hybrid protein molecule of 135 amino acids long.

What is of particular interest in this construct of the invention is that in addition to the sub-units from rheumatogenic end terminal fractions, a non-rheumatogenic serotype of streptococci was encoded and expressed, namely, that of M12.

This is an important aspect of the invention which is not limited to a particular non-rheumatogenic M12 serotype of streptococci as illustrated. Indeed, instead of a fraction of M12 an appropriate fraction such as of 10, 12 or more amino acids of any of the non-rheumatogenic serotypes can be used such as the following: 2, 10, 8, 9, 11, 22, 33 and others.

While a certain number of the rheumatogenic type M proteins have not yet been sequenced or their sequence not yet disclosed, such sequencing is readily performed by methods known in the art and hence appropriate fractions free of epitopes that cross-react with human tissue be made to constitute the multivalent hybrid of the invention. It is also contemplated that more than one fraction of the rheumatogenic serotype of streptococci be contained in the multivalent vaccine. When the antigenicity of this octavalent hybrid protein is tested, it reacts with polyclonal rabbit antisera raised against each component of the hybrid protein. Its immunogenicity and lack of cross-reactivity with human tissue especially myocardium, can be tested in accordance with the assays described herein. Thus, the immune sera will obtain opsonic antibodies against all seven types of serotypes of group A streptococci. The immunogenicity of the tetravalent protein and the antibody level will be determined. The immune sera will also contain opsonic antibodies against all four serotypes of group A streptococci will be tested for bacterial activity in vitro on an in vivo passive mouse protection tests as described. The multivalent protein is assayed in accordance with the assay described herein for the presence of broadly protective antibodies using passive mouse protection assays. In this case actively immunized mice will be similarly challenged with the virulent streptococci.

Thus in accordance with the invention, a multivalent hybrid M protein is provided which provides broad immunity against several serotypes and also elicit protective mucosal immunity.

It is also the contemplation of the invention to have a multivalent vaccine constituted of two different serotypes followed by a non-rheumatogenic fraction as was illustrated herein above, for example in conjunction with FIG. 13. Likewise, it should be noted that it is conceivable that the hybrid gene and hence the expressed hybrid protein have the non-rheumatogenic serotype of the streptococcus as the first fraction upstream of the rheumatogenic amino acid fractions of the amino terminal portions of the respective serotypes of streptococci. Further, it is within the contemplation of the invention to construct hybrid genes and the expressed hybrid protein constituted of one rheumatogenic serotype, such as M24-M5-M6-M19-M1-M3-M18 or others followed or preceded by a non-rheumatogenic fraction of a serotype of streptococci, such as M12 or others.

Of particular interest in conjunction with the invention are vaccines which include amino acid sub-units of any or all of the 1 through 80 different serotypes known or to be discovered of which approximately 15 are known to be causative or at least to contribute in the development of acute rheumatic fever following strep throat.

It is important to note that the invention is not limited to a particular amino acid sequence wherever herein amino acid sequences are referred to or described. In any particular amino acid sequence or fragment referred to herein, any one or more of amino acids can be removed, substituted, i.e. replaced by some other amino acid(s) as long as the desired epitopes are not adversely affected by such changes in the structure of the amino acid. Indeed this is quite commonly found in that the amino acid sequence of certain types of M proteins such as type M5 which originates from different strains of M5 (and also come from different origins and/or at different times) may have different amino acids substitutions, i.e. constitution. This has been shown for several such M proteins. Reference to that effect should be made to Miller et al., J. Biol. Chem., 263: 5668 (1988) "Antigenic Variation Among Group A Streptococcal M Proteins: Nucleotide Sequence of the Serotype 5M Protein Gene and its Relationship with Genes Encoding Types 1, 6 and 24 Proteins" and also see Dale et al., J. Exp. Med., 163:1191-1202 (1986), "Localization of Protective Epitopes of the Amino Terminus of Type 5 Streptococcal M Protein".

As has been described the N-terminal segment which is free of tissue cross-reactive epitopes can range from 10 to 115 amino acids and as an average be about 35 amino acids depending on the particular M protein type.

Accordingly, any single fragment of sub-unit constituting the hybrid gene and hence the expressed hybrid protein can be constructed to have a number of amino acid substitutions so as to contains such amino acid substitutions from one strain and for instance, two or more substitutions of another strain of the same serotype. Thus, functionally the antibodies generated would react optimally from and with all the strains of the particular type 5M protein.

It is therefore an important concept in this invention that when reference is made to a particular serotype (i.e. of anyone of the known or to be discovered serotypes e.g., 1-82) reference is not intended to one single type or strain such as that of M5-M6-M19-M24, but to the various strains of such serotypes which may as described, have amino acid variants. Thus, not only is the fundamental concept of the invention to provide a multivalent vaccine against different serotypes, but also different strains within that particular serotype.

Likewise, the nucleotide sequences can be so modified to code for the desired immunobiologically functionally equivalent amino acid sequences. Similarly, it is within the scope of the invention that due to degeneracy of the genetic code DNA sequences be constructed or used that encode and express the desired amino acid fragments in a selected organism transformed (or transfected) with the selected self replicating vehicle.

As has been described herein the invention is not limited to a particular maximum of multivalent hybrid gene or expressed multivalent vaccine by any specific number of serotypes. Since however there appear to be practical limits, it had been suggested that cocktail or mixture of appropriately sized multivalent hybrids be constructed.

Another aspect of the present invention are hybrid or fusion genes which have been constructed which encode the antigens of the present invention. The fusion genes code for the antigens of the invention, constituted as described above, of amino acid fragments linked

to the selected carrier. The genes are inserted into suitable self-replicating vehicles, like plasmids. The plasmids containing the genes are then used to transform nonvirulent microorganisms. The transformed microorganisms express the hybrid or fusion protein antigens which are capable of eliciting opsonic and/or protective antibodies against serotypes of Group A streptococcus in immunized mammals, without eliciting cross-reactive antibodies to mammalian heart tissue antigens.

The compositions of the invention can be administered by any suited route, including orally and nasally. They can be dispersed in an appropriate propellant, as for nasal administration.

The therapeutic compositions of the present invention may also be administered parenterally. Mammals, in particular humans, immunized parenterally with a sufficient amount of the therapeutic composition of the present invention develop opsonic and/or protective antibodies directed to the epitopes of the hybrid streptococcal M protein antigen. Non-limiting examples of such parenteral routes of administration are intracutaneous and intramuscular.

For intracutaneous injection, 100-300 μg of hybrid antigen emulsified in complete or incomplete Freund's adjuvant was administered in a mammal. A booster injection of about the same dose in saline was administered about one month later. Blood was obtained prior to the first injection and at two-week intervals thereafter for eight weeks.

A topical method of administration is also provided, namely intranasal. For intranasal administration, a mammal received about 50 μg to about 10 mg of purified antigen in an appropriate diluent for administration. Such method may be particularly well suited when the vaccine is constructed to evoke secretory or mucosal immunity since nasopharyngeal infection is a common infection in humans.

In accordance with the invention, the therapeutic composition may be administered singly in series or advantageously in a mixture or cocktail of multiple compositions to elicit broad spectrum immunity versus Group A streptococci.

The vaccine compositions of the invention which include the antigens of the invention may be adminsitered as disclosed in U.S. Patent No. 5,124,153 to BEachey et al., which is incororporated herein by reference and optionally, biologically acceptable diluents or adjuvant. The compositions are suitable for eliciting opsonic and/or protective antibodies to serotypes of M protein of Group A streptococcus. The administered compositions of the present invention elicit such antibodies, without eliciting cross-reactive antibodies to mammalian heart tissue antigens.

The plasmids which encode the M protein hybrid genes of the present invention may be cloned first and expressed in Escherichia coli. Any other enteric bacilli of the coliform group such as Klebsiella or Enterobacter can be used, but normally E. coli is preferred. The plasmid carrying the hybrid M gene is isolated and purified and then a construct is built to transform the desired avirulent bacteria, such as the araA-S. typhimurium (SL3261). This mutant strain exhibits a nutritional marker both for PABA and 2,3-DHB. See Brown et al., (in Beachey patent). Another desired specie of S. typhimurium is recA-S. typhimurium, particularly strain Ty21a. See Clements, et al., "Construction of a Potential Live Aro Vaccine for typhoid type fever and cholorea-E. coli-related diarrheas", Infect. Immun., 46:564-9 (1984). Also see the other references cited in the above cited Brown, et al., article, which are also incorporated herein by reference. Vectors that can be transformed in host cells of other gram negative bacteria such as of the Enterobacteriaceae genus (such as Shigella and Klebsiella like (Klebsiella pneumonia; Enterobacter like Enterobacter aerogenes). Salmonellae, such as Salmonella arizona, and Citrobacter may be used if appropriately rendered non-virulent or attenuated. Common Salmonella species which may be used when attenuated and rendered non-virulent include the following: S. paratyphi A, S. schottmulleri, S. typhimurium, S. paratyphi C, S. choleraesuis, S. montevideo, S. newport, S. typhi, S. enteritidis, S. gallinarum, and S. anatum.

There may also be used as host bacteria of the Streptococcus genus which are non-virulent or which have been made non-virulent or attenuated, including streptococci of the immunological groups A-O generally other than A. Suitable Streptococci which can be used as

bacterial host include S. cremoris, S. faecalis, S. salivarius, S. mitior, S. mitis, S. mutans and S. sanguis. Particularly preferred are S. sanguis, S. mutans, which is non-cariogenic.

Additional appropriate microorganisms which may be attenuated and transformed in accordance with the invention are known. Reference may be made to Davis, et al., Microbiology, (Harper & Row, Second edition, 1973).

Generally any enteric bacterium may serve as the host bacterium. It is preferable that the host bacterium only survive in the subject long enough to elicit the opsonic response, but generally any bacterial strain that has been attenuated so as not to colonize yet still multiply to a limited degree to elicit antibodies to the protein antigen of the present invention can be used. In a preferred embodiment of the invention the Aro$^-$ strain of S. typhimurium is used, which requires two metabolites not found in mammalian tissues, PABA and 2,3-DHB. As a result, the inoculated bacteria die after several generations from a lack of these metabolites. See Hoiseth and Stocker, (in Beachey patent). However, any mutated microbial agent with a metabolic deficiency for nutritional compounds not found in the tissues of the subject to be immunized, or one so made by genetic manipulations, may be employed. The expression of the hybrid gene is confined almost exclusively to cytoplasmic compartment.

In accordance with the present invention, general molecular biology methods are used. Any suitable plasmid or bacteriophage cloning vector may be selected. The vector should have an origin of replication that is functional in the intended microbial host cells, and a selectable marker (such as an antibiotic resistance gene) to aid in identification of host cells that have been transformed with the vector. It should be able to accept inserted DNA fragments and still replicate normally. Preferably, the vector comprises one or more unique restriction endonuclease recognition sites at which hybrid DNA fragments can be inserted without destroying the vector's ability to replicate.

Suitable cloning vectors include phage derivatives such as lambda gt11 (Young and Davis, Proc. Natl. Acad. Sci. USA, 80, 1194-1198 (1983)), the various phage M13-derived vectors such as M13mp9 (commercially available from Bethesda Research Labs), plasmids such as pBR322,

and many others (Old and Primose, Principles of Gene Manipulation, 2nd Ed., University of California, Press, 32-35 and 46-47 (1981)). The streptococcal DNA is inserted into the cloning vector by such standard methods as homopolymeric tailing or by using linker molecules.

In connection with the invention as described, the PCR method and other molecular biology and immunology methods and materials are used. But for the method for synthesizing particular fragments in the pre-selected sequence and orientation, the PCR method and other materials used herein are described in several general standard texts and laboratory manuals. For instance, Sambrook, section 14, in vitro Amplification of DNA by PCR; Ausbel Protocols Molecular Biology, Section 15; for protein expression see same, section 16; for prokaryote and eukaryote expression vectors, see Sambrook, Section 1.7; Protocols Molecular, Section 1, e.g. E. coli Plasmids, listing numerous available plasmids. For other suitable vectors for molecular cloning, see Perbal (2nd Ed.), Section 6, which lists for instance, cloning vectors desired from pBR322 (used herein). For material, protocols, etc. in immunology, see in general Current Protocols, Immunology; also see Section 7 for Immunologic Studies in Humans and Section 8 for Isolation and Analysis of Proteins. The ATCC Catalogue of Bacteria and Phages lists suitable microorganisms. For a catalogue of Yeasts, see ATCC Catalogue of Yeast (1990), 18th Ed. For available Recombinant DNA Materials (Hosts, Libraries, Vectors, Clones, etc.), see ATCC catalogue of Recombinant DNA Materials 2nd Ed. (1991).

Another worthwhile publication is Immunology of Proteins, Atassi (vol. 3), Plenium Press (1979).

This invention makes a significant contribution to the medical arts. It is contemplated to be within the scope of the invention that substantially the same results be obtained by substantially the same means operating or performing in substantially the same manner as described herein.

One skilled in the art may refer to the below listed bibliography which is incorporated herein by reference.

Table 1. Inhibition of opsonization of types 24, 5, and 6 streptococci by M24-M5-M6 trivalent hybrid M protein.

Serotype	Antiserum	Inhibitor	Precent Opsonization
M24	preimmune	-	0
	anti-pep M24	-	78
		M24-M5-M6	0
		pep M24	0
M5	preimmune	-	6
	anti SM5(1-15)	-	64
		M24-M5-M6	16
		pep M5	6
M6	preimmune	-	0
	anti SM6(1-20)	-	58
		M24-M5-M6	4
		pep M6	0

Table 2. Immunogenicity of M24-M5-M6 trivalent hybrid M protein in rabbits

Rabbit Number		ELISA titer against:					
		M24-M5-M6	pep M24	SM5(1-15)C	pep M5	SM6(1-20)C	pep M6
9140	Preimmune	<100	<100	<100	<100	<100	<100
	16 wks	25,600	6,400	3,200	3,200	6,400	800
9141	Preimmune	<100	<100	<100	<100	<100	<100
	16 wks	51,200	1,600	3,200	800	3,200	800
9142	Preimmune	<100	<100	<100	<100	<100	<100
	16 wks	25,600	1,600	3,200	400	1,600	800

Table 3. Opsonic antibodies evoked in rabbits by SM24-M5-M6 hybrid M proteins

Antisera	Percent opsonization of: Type 24 streptococci	Type 5 streptococci	Type 6 streptococci
Preimmune pool	2	8	6
9140	94	70	4
9141	84	24	4
9142	58	10	4
Anti pep M24	98	N.D.	N.D.
Anti pep M5	N.D.	96	N.D.
Anti pep M6	N.D.	N.D.	94

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We claim:

1. An immunogenic recombinant multivalent hybrid M protein which comprises amino acid fragments of streptococcal M protein that carry epitopes that elicit opsonic antibodies against multiple serotypes of Group A streptococci, said protein not eliciting tissue cross-reactive antibodies.

2. The immunogenic recombinant multivalent hybrid M protein of claim 1, wherein the amino acid fragments are amino-terminal fragments of the streptococcal M protein.

3. The immunogenic recombinant multivalent hybrid M protein of claim 2, which hybrid M protein also elicits mucosal antibodies.

4. The immunogenic multivalent hybrid M protein of claim 2, wherein the amino acid fragments comprise amino-terminal portions of serotypes of potentially rheumatogenic streptococci serotypes.

5. The immunogenic recombinant multivalent hybrid M protein of claim 4, wherein the serotypes are selected from the group of serotypes M1, M3, M12, M18, M19 and M24.

6. The immunogenic recombinant multivalent hybrid M protein of claim 5, which is tetravalent.

7. The immunogenic recombinant multivalent hybrid M protein of claim 5, which is trivalent.

8. The immunogenic recombinant multivalent hybrid M protein of claim 7, wherein the amino acid fragments are selected from the group consisting of M24, M5 and M19.

9. The immunogenic recombinant multivalent hybrid M protein of claim 6, wherein the amino acids are selected from the group consisting of M24, M5, M6 and M19.

10. The immunogenic recombinant multivalent hybrid M protein of claim 6, wherein the M24 fragment is 113 amino acids long, the M5 fragment is 58 amino acid long, the M6 is 35 amino acid long and the M19 fragment is 35 amino acid long.

11. The immunogenic recombinant multivalent hybrid M protein of claim 7, wherein the M24 fragment is 260 amino acid long, the M5 fragment is 11 amino acid long and the M6 fragment is 12 amino acid long.

12. The immunogenic recombinant multivalent hybrid M protein of claim 3, which comprises amino acids of the C-repeats of the M protein.

13. The immunogenic recombinant multivalent hybrid M protein of claim 12, which comprises a carboxyl terminal fragment of an M protein.

14. The immunogenic recombinant multivalent hybrid M protein of claim 1, wherein amino acid fragments are fused to each other by a linker comprising amino acids.

15. The therapeutic recombinant multivalent hybrid M protein of claim 14, wherein the linkers of the amino acids are selected from the group consisting of Arg, Ser, Val, Asp, Pro, and Trp.

16. An immunogenic composition which comprises a therapeutically acceptable carrier and the protein of claim 1.

17. A method for immunizing a mammal against streptococci infections by eliciting opsonic antibodies to a streptococcal M protein without eliciting antibodies which are cross-reactive with heart tissue antigens which comprises administering to said mammal an amount effective to confer immunity against group A streptococci infections, the therapeutic composition of claim 16.

18. A hybrid recombinant DNA which comprises fused nucleotide sequences of streptococcal M protein which encode amino acid fragments of streptococcal M protein that carry epitopes that elicit opsonic antibodies against multiple serotypes of group A streptococci.

19. The hybrid recombinant DNA of claim 18 which comprises also nucleotide sequences which encode C-terminal amino acids of the M protein which evoke mucosal antibodies.

20. The hybrid recombinant DNA of claim 19, wherein the nucleotide sequence encodes amino acids of the C-terminal repeats of the M protein.

Figure 1

RECOMBINANT M24-M5-M6

```
          10         20         30         40         50         60
ATG GTC GCG ACT AGG TCT CAG ACA GAT ACT CTG GAA AAA GTA CAA GAA CGT GCT GAC AAG
  Val Ala Thr Arg Ser Gln Thr Asp Thr Leu Glu Lys Val Gln Glu Arg Ala Asp Lys
M24 ------>

          70         80         90         100        110        120
TTT GAG ATA GAA AAC AAT ACG TTA AAA CTT AAG AAT AGT GAC TTA AGT TTT AAT AAT AAA
Phe Glu Ile Glu Asn Asn Thr Leu Lys Leu Lys Asn Ser Asp Leu Ser Phe Asn Asn Lys

          130        140        150        160        170        180
GCG TTA AAA GAT CAT AAT GAT GAG TTA ACT GAA GAG TTG AGT AAT GCT AAA GAG AAA CTA
Ala Leu Lys Asp His Asn Asp Glu Leu Thr Glu Glu Leu Ser Asn Ala Lys Glu Lys Leu

          190        200        210        220        230        240
CGT AAA AAT GAT AAA TCA CTA TCT GAA AAA GCT AGT AAA ATT CAA GAA TTA GAG GCA CGT
Arg Lys Asn Asp Lys Ser Leu Ser Glu Lys Ala Ser Lys Ile Gln Glu Leu Glu Ala Arg

          250        260        270        280        290        300
AAG GCT GAT CTT GAA AAA GCA TTA GAA GGC GCA ATG AAT TTT TCA ACA GCG GAT TCA GCT
Lys Ala Asp Leu Glu Lys Ala Leu Glu Gly Ala Met Asn Phe Ser Thr Ala Asp Ser Ala

          310        320        330        340        350        360
AAA ATC AAA ACC TTA GAA GCA GAG AAA GCT GCT TTA GCG GCA CGT AAG GCT GAT CTT GAA
Lys Ile Lys Thr Leu Glu Ala Glu Lys Ala Ala Leu Ala Ala Arg Lys Ala Asp Leu Glu

          370        380        390        400        410        420
AAA GCA TTA GAA GGC GCA ATG AAC TTT TCA ACA GCG GAT TCA GCT AAA ATC AAA ACC TTA
Lys Ala Leu Glu Gly Ala Met Asn Phe Ser Thr Ala Asp Ser Ala Lys Ile Lys Thr Leu

          430        440        450        460        470        480
GAA GCA GAG AAA GCT GCT TTA GAG GCA CGC CAG GCT GAA CTT GAA AAA GCA TTA GAA GGC
Glu Ala Glu Lys Ala Ala Leu Glu Ala Arg Gln Ala Glu Leu Glu Lys Ala Leu Glu Gly

          490        500        510        520        530        540
GCA ATG AAT TTT TCA ACA GCG GAT TCA GCT AAA ATC AAA ACC TTA GAA GCA GAG AAA GCT
Ala Met Asn Phe Ser Thr Ala Asp Ser Ala Lys Ile Lys Thr Leu Glu Ala Glu Lys Ala

          550        560        570        580        590        600
GCT TTA GCG GCA CGT AAG GCT GAT CTT GAA AAA GCA TTA GAA GGC GCA ATG AAC TTT TCA
Ala Leu Ala Ala Arg Lys Ala Asp Leu Glu Lys Ala Leu Glu Gly Ala Met Asn Phe Ser

          610        620        630        640        650        660
ACA GCG GAT TCA GCT AAA ATC AAA ACC TTA GAA GCA GAG AAA GCT GCT TTA GAG GCA CGC
Thr Ala Asp Ser Ala Lys Ile Lys Thr Leu Glu Ala Glu Lys Ala Ala Leu Glu Ala Arg

          670        680        690        700        710        720
CAG GCT GAA CTT GAA AAA GCA TTA GAA GGC GCA ATG AAT TTT TCA ACA GCG GAT TCA GCT
Gln Ala Glu Leu Glu Lys Ala Leu Glu Gly Ala Met Asn Phe Ser Thr Ala Asp Ser Ala

          730        740        750        760        770        780
AAA ATC AAA ACC TTA GAA GCA GAG AAA GCT GCT TTG GAG GCA GAG AAA GCT GAT CTT GAA
Lys Ile Lys Thr Leu Glu Ala Glu Lys Ala Ala Leu Glu Ala Glu Lys Ala Asp Leu Glu

Bam H1    790        800        810        820 Sal I  830        840
GGA TCC GCC GTG ACT AGG GGT ACA ATA AAT GAC CCG CAA GTC GAC AGA GTG TTT CCT AGG
Arg Ser Ala Val Thr Arg Gly Thr Ile Asn Asp Pro Gln Val Asp Arg Val Phe Pro Arg
      M5 ------------------------------------------------------>  M6 ------>
          850        860
GGG ACG GTA GAA AAC CCG GAC
Gly Thr Val Glu Asn Pro Asp
------------------------>
```

Figure 2

IMMUNOBLOT ANALYSIS OF rM24.M5.M6 HYBRID VACCINE

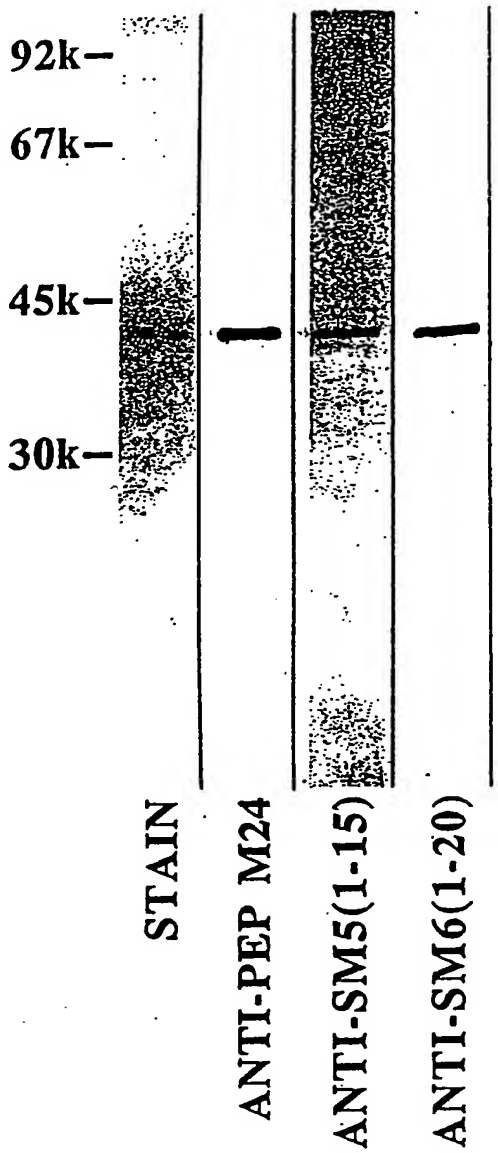
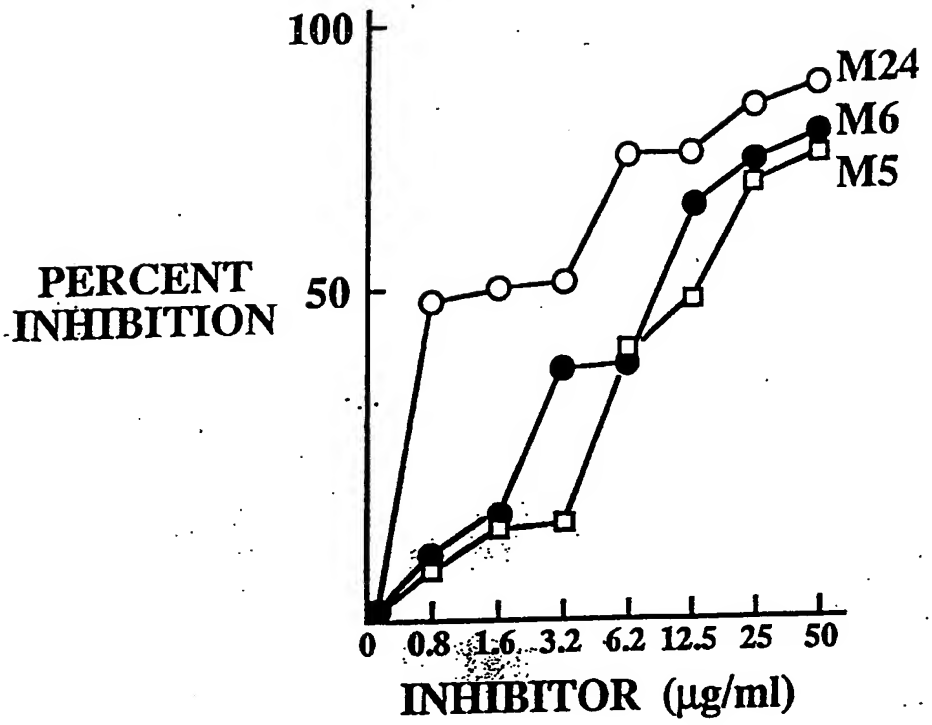


Figure 3


100
50
PERCENT INHIBITION
M24
M6
M5
0 0.8 1.6 3.2 6.2 12.5 25 50
INHIBITOR (µg/ml)

WO 94/06421

Figure 4

RECOMBINANT M24-M5-M6-M19

M24 →

```
          10         20         30         40         50         60
ATG GTC GCG ACT AGG TCT CAG ACA GAT ACT CTG GAA AAA GTA CAA GAA CGT GCT GAC AAG
Met Val Ala Thr Arg Ser Gln Thr Asp Thr Leu Glu Lys Val Gln Glu Arg Ala Asp Lys

          70         80         90        100        110        120
TTT GAG ATA GAA AAC AAT ACG TTA AAA CTT AAG AAT AGT GAC TTA AGT TTT AAT AAT AAA
Phe Glu Ile Glu Asn Asn Thr Leu Lys Leu Lys Asn Ser Asp Leu Ser Phe Asn Asn Lys

         130        140        150        160        170        180
GCG TTA AAA GAT CAT AAT GAT GAG TTA ACT GAA GAG TTG AGT AAT GCT AAA GAG AAA CTA
Ala Leu Lys Asp His Asn Asp Glu Leu Thr Glu Glu Leu Ser Asn Ala Lys Glu Lys Leu

         190        200        210        220        230        240
CGT AAA AAT GAT AAA TCA CTA TCT GAA AAA GCT AGT AAA AAT CAA GAA TTA GAG GCA CGT
Arg Lys Asn Asp Lys Ser Leu Ser Glu Lys Ala Ser Lys Asn Gln Glu Leu Glu Ala Arg

         250        260        270        280        290        300
AAG GCT GAT CTT GAA AAA GCA TTA GAA GGC GCA ATG AAT TTT TCA ACA GCG GAT TCA GCT
Lys Ala Asp Leu Glu Lys Ala Leu Glu Gly Ala Met Asn Phe Ser Thr Ala Asp Ser Ala
                                                  BamH1 M5 →
         310        320        330        340        350        360
AAA ATC AAA ACC TTA GAA GCA GAG AAA GCT GAT CTT GAA GGA TCC GCC GTG ACT AGG GGT
Lys Ile Lys Thr Leu Glu Ala Glu Lys Ala Asp Leu Glu Gly Ser Ala Val Thr Arg Gly

         370        380        390        400        410        420
ACA ATA AAT GAC CCG CAA AGA GCA AAA GAA GCT CTT GAC AAG TAT GAG CTA GAA AAC CAT
Thr Ile Asn Asp Pro Gln Arg Ala Lys Glu Ala Leu Asp Lys Tyr Glu Leu Glu Asn His

         430        440        450        460        470        480
GAC TTA AAA ACT AAG AAT GAA GGG TTA AAA ACT GAG AAT GAA GGG TTA AAA ACT GAG AAT
Asp Leu Lys Thr Lys Asn Glu Gly Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu Asn
                                                  Sal1 M6 →
         490        500        510        520        530        540
GAA GGG TTA AAA ACT GAG AAT GAA GGG TTA AAA ACT GAG GTC GAC AGA GTG TTT CCT AGG
Glu Gly Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu Val Asp Arg Val Phe Pro Arg

         550        560        570        580        590        600
GGG ACG GTA GAA AAC CCG GAC AAA GCA CGA GAA CTT CTT AAC AAG TAT GAC GTA GAG AAC
Gly Thr Val Glu Asn Pro Asp Lys Ala Arg Glu Leu Leu Asn Lys Tyr Asp Val Glu Asn
                                  Nco1 M19 →
         610        620        630        650        660
TCT ATG TTA CAA GCT AAT AAT GAC AAC TTA CCA TGG AGA GTG CGT TAT ACT AGG CAT ACG
Ser Met Leu Gln Ala Asn Asn Asp Asn Leu Pro Trp Arg Val Arg Tyr Thr Arg His Thr

         670        680        690        700        710        720
CCA GAA GAT AAG CTA AAA AAA ATT ATT GAC GAT CTT GAC GCA AAA GAA CAT GAA TTA CAA
Pro Glu Asp Lys Leu Lys Lys Ile Ile Asp Asp Leu Asp Ala Lys Glu His Glu Leu Gln

         730        740
CAA CAG AAT GAG AAG TTA TCT
Gln Gln Asn Glu Lys Leu Ser
```

Figure 5

IMMUNOBLOT ANALYSIS OF rM24.M5.M6.M19 HYBRID VACCINE

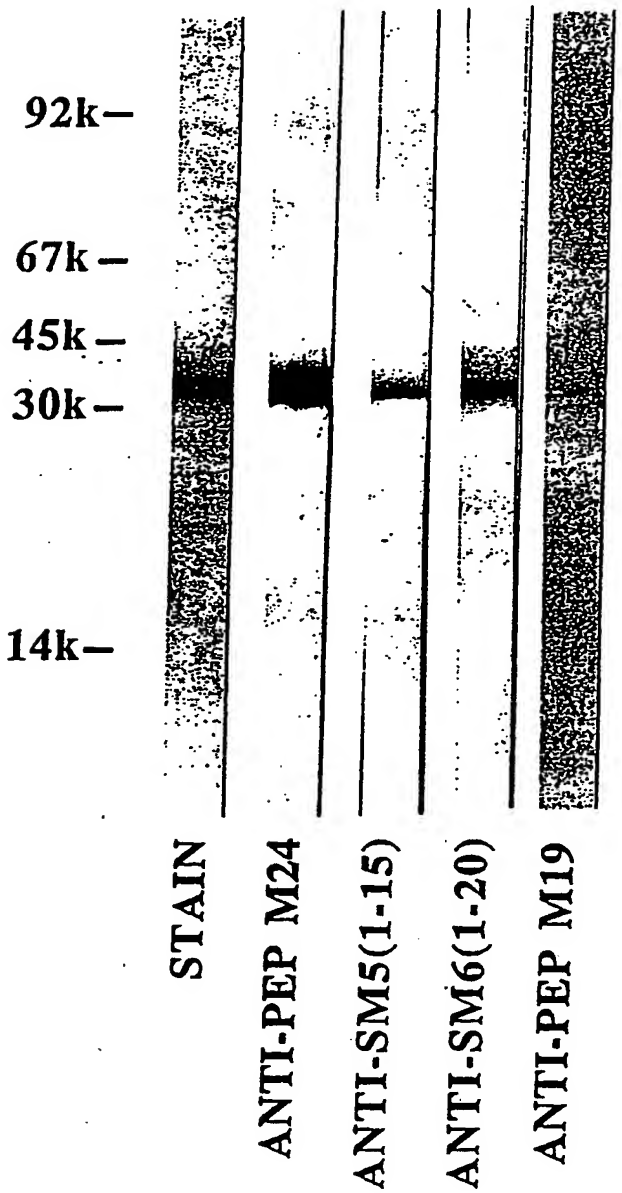


Figure 6

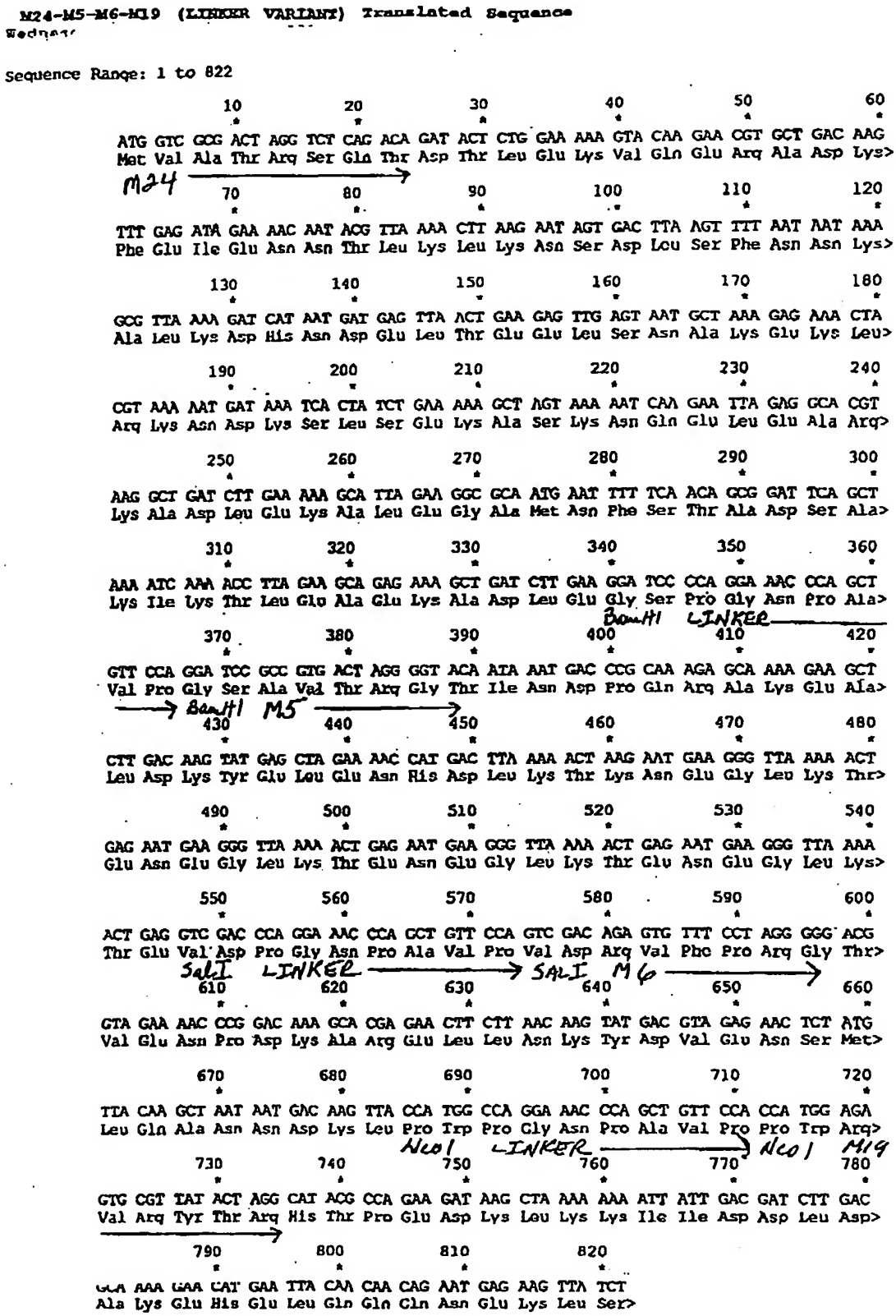

M24-M5-M6-M19 (LINKER VARIANT) Translated Sequence
Sequence Range: 1 to 822
M24
BamHI
LINKER
BamHI M5
SalI LINKER
SalI M6
NcoI LINKER
NcoI M19

Figure 7

M24-M5-M6-M19 (SUBUNIT VAR) Translated Sequence

Sequence Range: 1 to 561

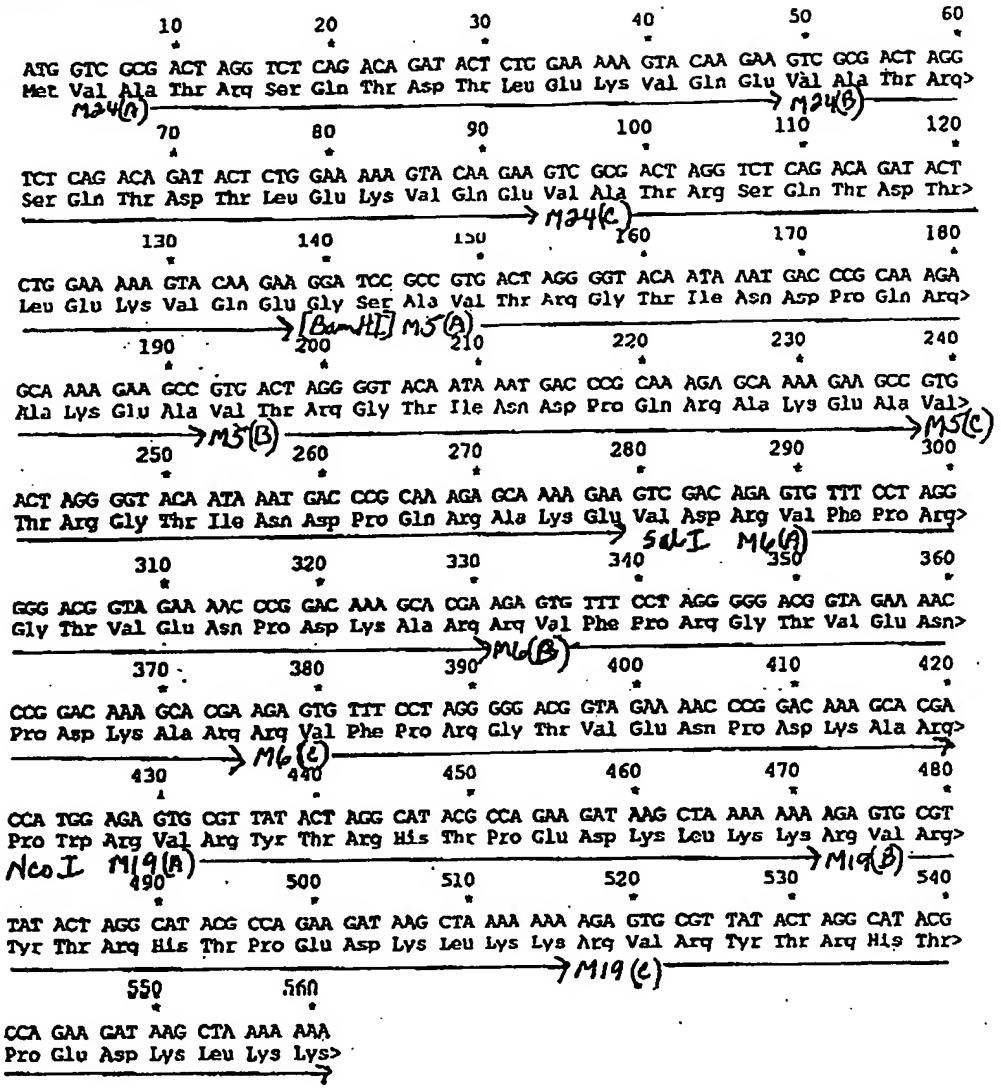
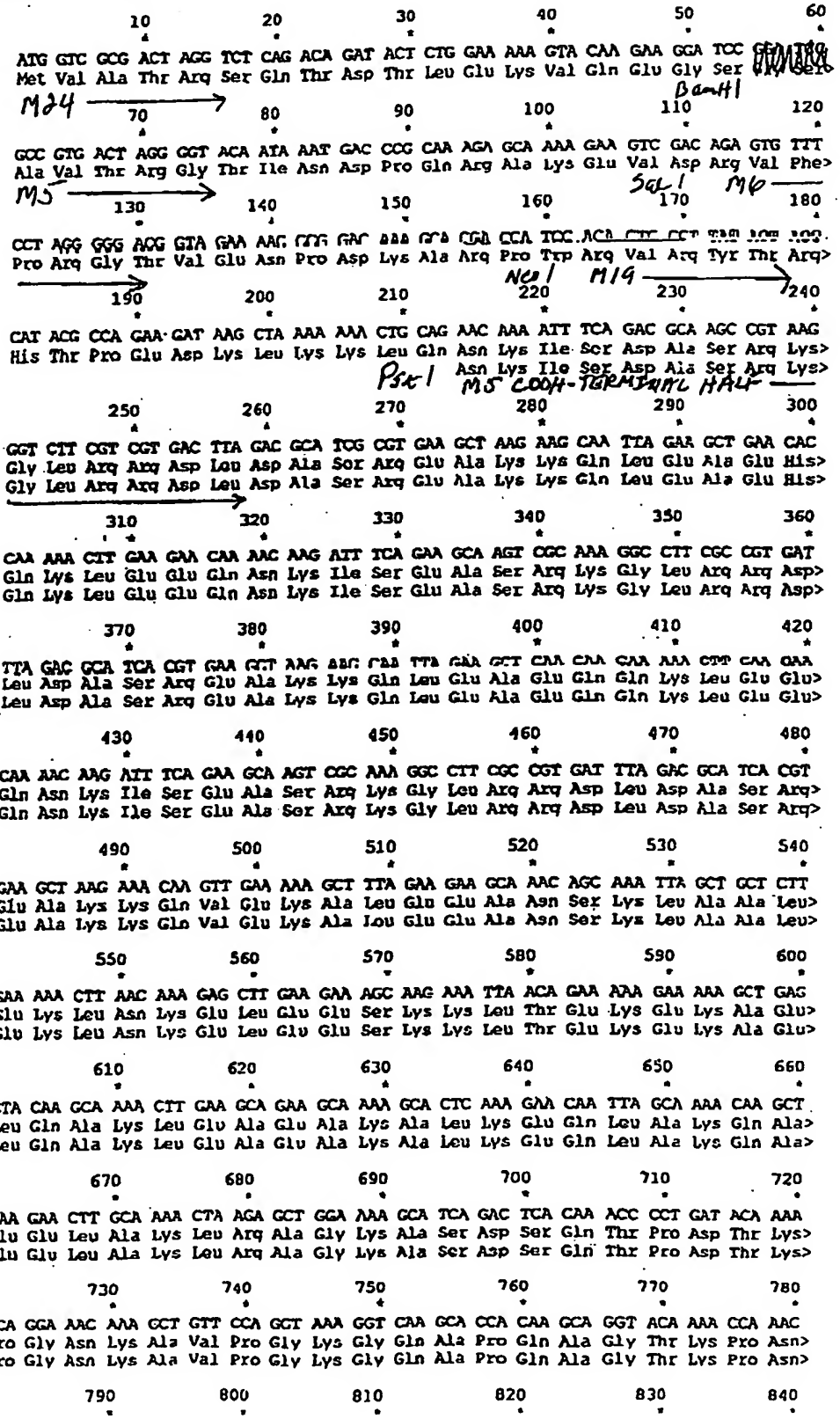


Figure 8

M24-5-6-19-C-TERM Translated Sequence

Sequence Range: 1 to 924



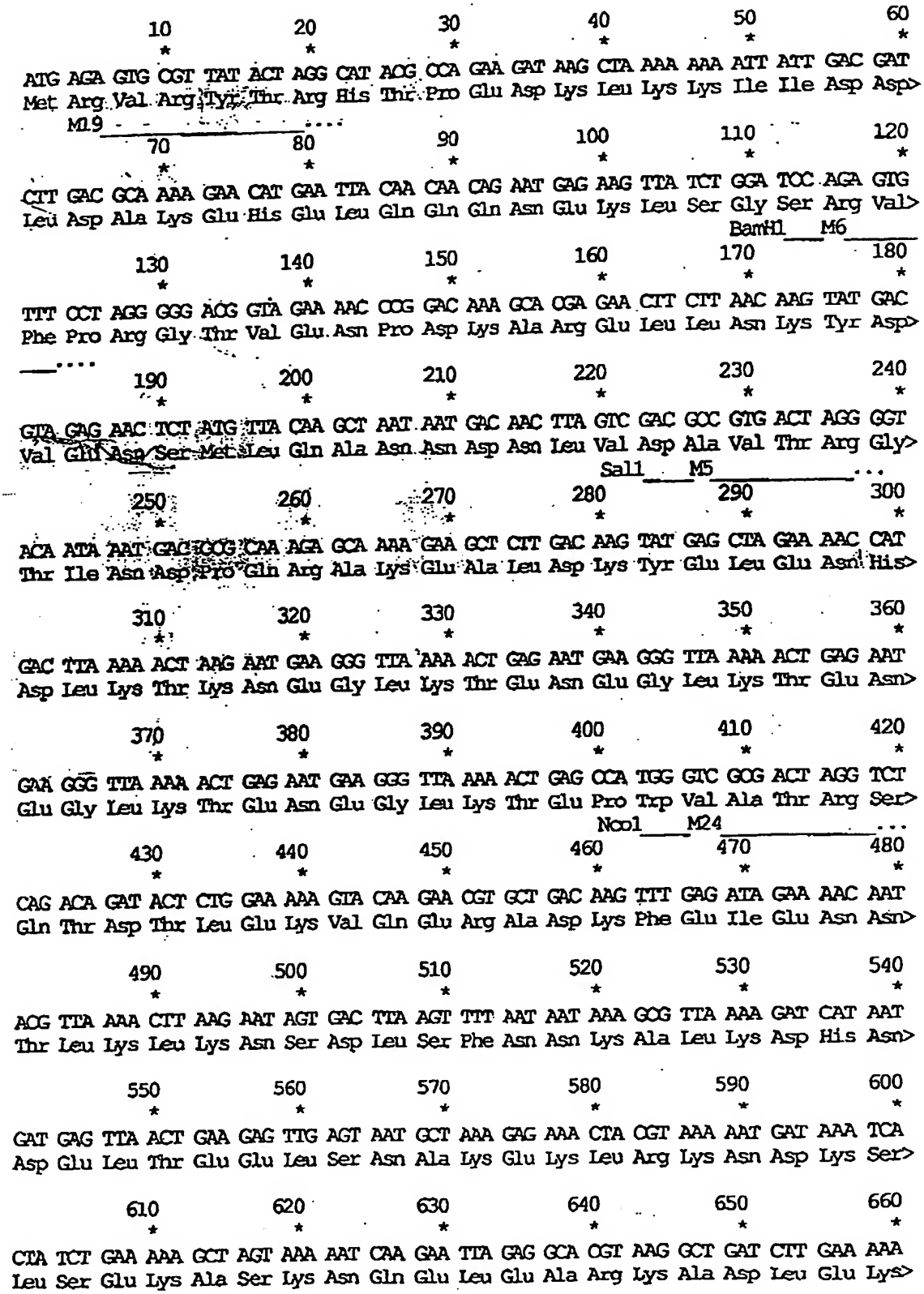
```
CAA AAC AAA GCA CCA ATG AAG GAA ACT AAG AGA CAG TTA CCA TCA ACA GGT GAA ACA GCT
Gln Asn Lys Ala Pro Met Lys Glu Thr Lys Arg Gln Leu Pro Ser Thr Gly Glu Thr Ala>
Gln Asn Lys Ala Pro Met Lys Glu Thr Lys Arg Gln Leu Pro Ser Thr Gly Glu Thr Ala>

          850         860         870         880         890         900
           *           *           *           *           *           *
AAC CCA TTC TTC ACA GCG GCA GCC CTT ACT GTT ATG GCA ACA GCT GGA GTA GCA GCA GTT
Asn Pro Phe Phe Thr Ala Ala Ala Leu Thr Val Met Ala Thr Ala Gly Val Ala Ala Val>
Asn Pro Phe Phe Thr Ala Ala Ala Leu Thr Val Met Ala Thr Ala Gly Val Ala Ala Val>

          910         920
           *           *
GTA AAA CGC AAA GAA GAA AAT TAA
Val Lys Arg Lys Glu Glu Asn ***>
Val Lys Arg Lys Glu Glu Asn ***>
```

Figure 9

M19-M6-M5-M24 TETRAVALENT HYBRID



```
          670         680         690         700         710         720
           *           *           *           *           *           *
GCA TTA GAA GGC GCA ATG AAT TTT TCA ACA GCG GAT TCA GCT AAA ATC AAA ACC TTA GAA
Ala Leu Glu Gly Ala Met Asn Phe Ser Thr Ala Asp Ser Ala Lys Ile Lys Thr Leu Glu>

          730         740
           *           *
GCA GAG AAA GCT GAT CTT GAA-
Ala Glu Lys Ala Asp Leu Glu>
```

Figure 10

M24-M5 DIVALENT Translated Sequence

Sequence Range: 1 to 522

```
                10          20          30          40          50          60
                *           *           *           *           *           *
ATG GTC GCG ACT AGG TCT CAG ACA GAT ACT CTG GAA AAA GTA CAA GAA CGT GCT GAC AAG
Met Val Ala Thr Arg Ser Gln Thr Asp Thr Leu Glu Lys Val Gln Glu Arg Ala Asp Lys>
    M24 ------>
                70          80          90          100         110         120
                *           *           *           *           *           *
TTT GAG ATA GAA AAC AAT ACG TTA AAA CTT AAG AAT AGT GAC TTA AGT TTT AAT AAT AAA
Phe Glu Ile Glu Asn Asn Thr Leu Lys Leu Lys Asn Ser Asp Leu Ser Phe Asn Asn Lys>

            130         140         150         160         170         180
            *           *           *           *           *           *
GCG TTA AAA GAT CAT AAT GAT GAG TTA ACT GAA GAG TTG AGT AAT GCT AAA GAG AAA CTA
Ala Leu Lys Asp His Asn Asp Glu Leu Thr Glu Glu Leu Ser Asn Ala Lys Glu Lys Leu>

            190         200         210         220         230         240
            *           *           *           *           *           *
CGT AAA AAT GAT AAA TCA CTA TCT GAA AAA GCT AGT AAA AAT CAA GAA TTA GAG GCA CGT
Arg Lys Asn Asp Lys Ser Leu Ser Glu Lys Ala Ser Lys Asn Gln Glu Leu Glu Ala Arg>

            250         260         270         280         290         300
            *           *           *           *           *           *
AAG GCT GAT CTT GAA AAA GCA TTA GAA GGC GCA ATG AAT TTT TCA ACA GCG GAT TCA GCT
Lys Ala Asp Leu Glu Lys Ala Leu Glu Gly Ala Met Asn Phe Ser Thr Ala Asp Ser Ala>

            310         320         330         340         350         360
            *           *           *           *           *           *
AAA ATC AAA ACC TTA GAA GCA GAG AAA GCT GAT CTT GAA GGA TCC GCC GTG ACT AGG GGT
Lys Ile Lys Thr Leu Glu Ala Glu Lys Ala Asp Leu Glu Gly Ser Ala Val Thr Arg Gly>
                                                BAMHI  M5 ------>
            370         380         390         400         410         420
            *           *           *           *           *           *
ACA ATA AAT GAC CCG CAA AGA GCA AAA GAA GCT CTT GAC AAG TAT GAG CTA GAA AAC CAT
Thr Ile Asn Asp Pro Gln Arg Ala Lys Glu Ala Leu Asp Lys Tyr Glu Leu Glu Asn His>

            430         440         450         460         470         480
            *           *           *           *           *           *
GAC TTA AAA ACT AAG AAT GAA GGG TTA AAA ACT GAG AAT GAA GGG TTA AAA ACT GAG AAT
Asp Leu Lys Thr Lys Asn Glu Gly Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu Asn>

            490         500         510         520
            *           *           *           *
GAA GGG TTA AAA ACT GAG AAT GAA GGG TTA AAA ACT GAG TAA
Glu Gly Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu ***>
```

Figure 11

TETRAVALENT-C REPEAT Translated Sequence

Sequence Range: 1 to 1029

```
          10          20          30          40          50          60
          *           *           *           *           *           *
ATG AGA GTG CGT TAT ACT AGG CAT ACG CCA GAA GAT AAG CTA AAA AAA ATT ATT GAC GAT
Met Arg Val Arg Tyr Thr Arg His Thr Pro Glu Asp Lys Leu Lys Lys Ile Ile Asp Asp>
          M19 →
          70          80          90          100         110         120
          *           *           *           *           *           *
CTT GAC GCA AAA GAA CAT GAA TTA CAA CAA CAG AAT GAG AAG TTA TCT GGA TCT AGA GTC
Leu Asp Ala Lys Glu His Glu Leu Gln Gln Gln Asn Glu Lys Leu Ser Gly Ser Arg Val>
                                                    BamHI  M6 →
          130         140         150         160         170         180
          *           *           *           *           *           *
TTT CCT AGG GGG ACG GTA GAA AAC CCG GAC AAA GCA CGA GAA CTT CTT AAC AAG TAT GAC
Phe Pro Arg Gly Thr Val Glu Asn Pro Asp Lys Ala Arg Glu Leu Leu Asn Lys Tyr Asp>
          190         200         210         220         230         240
          *           *           *           *           *           *
GTA GAG AAC TCT ATG TTA CAA GCT AAT AAT GAC AAC TTA GTC GAC GCC GTG ACT AGG GGT
Val Glu Asn Ser Met Leu Gln Ala Asn Asn Asp Asn Leu Val Asp Ala Val Thr Arg Gly>
                                    SalI    M5 →
          250         260         270         280         290         300
          *           *           *           *           *           *
ACA ATA AAT GAC CCG CAA AGA GCA AAA GAA GCT CTT GAC AAG TAT GAG CTA GAA AAC CAT
Thr Ile Asn Asp Pro Gln Arg Ala Lys Glu Ala Leu Asp Lys Tyr Glu Leu Glu Asn His>
          310         320         330         340         350         360
          *           *           *           *           *           *
GAC TTA AAA ACT AAG AAT GAA GGG TTA AAA ACT GAG AAT GAA GGG TTA AAA ACT GAG AAT
Asp Leu Lys Thr Lys Asn Glu Gly Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu Asn>
          370         380         390         400         410         420
          *           *           *           *           *           *
GAA GGG TTA AAA ACT GAG AAT GAA GGG TTA AAA ACT GAG CCA TGG GTC GCG ACT AGG TCT
Glu Gly Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu Pro Trp Val Ala Thr Arg Ser>
                                        NcoI    M24 →
          430         440         450         460         470         480
          *           *           *           *           *           *
CAG ACA GAT ACT CTG GAA AAA GTA CAA GAA CGT GCT GAC AAG TTT GAG ATA GAA AAC AAT
Gln Thr Asp Thr Leu Glu Lys Val Gln Glu Arg Ala Asp Lys Phe Glu Ile Glu Asn Asn>
          490         500         510         520         530         540
          *           *           *           *           *           *
ACG TTA AAA CTT AAG AAT AGT GAC TTA AGT TTT AAT AAT AAA GCG TTA AAA GAT CAT AAT
Thr Leu Lys Leu Lys Asn Ser Asp Leu Ser Phe Asn Asn Lys Ala Leu Lys Asp His Asn>
          550         560         570         580         590         600
          *           *           *           *           *           *
GAT GAG TTA ACT GAA GAG TTG AGT AAT GCT AAA GAG AAA CTA CGT AAA AAT GAT AAA TCA
Asp Glu Leu Thr Glu Glu Leu Ser Asn Ala Lys Glu Lys Leu Arg Lys Asn Asp Lys Ser>
          610         620         630         640         650         660
          *           *           *           *           *           *
CTA TCT GAA AAA GCT AGT AAA AAT CAA GAA TTA GAG GCA CGT AAG GCT GAT CTT GAA AAA
Leu Ser Glu Lys Ala Ser Lys Asn Gln Glu Leu Glu Ala Arg Lys Ala Asp Leu Glu Lys>
          670         680         690         700         710         720
          *           *           *           *           *           *
GCA TTA GAA GGC GCA ATG AAT TTT TCA ACA GCG GAT TCA GCT AAA ATC AAA ACC TTA GAA
Ala Leu Glu Gly Ala Met Asn Phe Ser Thr Ala Asp Ser Ala Lys Ile Lys Thr Leu Glu>
          730         740         750         760         770         780
          *           *           *           *           *           *
GCA GAG AAA GCT GAT CTT GAA CGA TCG AAC AAA ATT TCA GAC GCA AGC CGT AAG GGT CTT
Ala Glu Lys Ala Asp Leu Glu Arg Ser Asn Lys Ile Ser Asp Ala Ser Arg Lys Gly Leu>
                                  Asn Lys Ile Ser Asp Ala Ser Arg Lys Gly Leu>
                                  M6 C TERM & REPEAT #1
          790         800         810         820         830         840
          *           *           *           *           *           *
CGT CGT GAC TTA GAC GCA TCG CGT GAA GCT AAG AAG CAA TTA GAA GCT GAA CAC CAA AAA
Arg Arg Asp Leu Asp Ala Ser Arg Glu Ala Lys Lys Gln Leu Glu Ala Glu His Gln Lys>
Arg Arg Asp Leu Asp Ala Ser Arg Glu Ala Lys Lys Gln Leu Glu Ala Glu His Gln Lys>
          850         860         870         880         890         900
          *           *           *           *           *           *
CTT GAA GAA CAA AAC AAG ATT TCA GAA GCA AGT CGC AAA GGC CTT CGC CGT GAT TTA GAC
Leu Glu Glu Gln Asn Lys Ile Ser Glu Ala Ser Arg Lys Gly Leu Arg Arg Asp Leu Asp>
Leu Glu Glu Gln Asn Lys Ile Ser Glu Ala Ser Arg Lys Gly Leu Arg Arg Asp Leu Asp>
                  ^ 2
```

TETRAVALENT-C REPEAT Translated sequence

```
          910         920         930         940         950         960
           *           *           *           *           *           *
GCA TCA CGT GAA GCT AAG AAG CAA TTA GAA GCT GAA CAA CAA AAA CTT GAA GAA CAA AAC
Ala Ser Arg Glu Ala Lys Lys Gln Leu Glu Ala Glu Gln Gln Lys Leu Glu Glu Gln Asn>
Ala Ser Arg Glu Ala Lys Lys Gln Leu Glu Ala Glu Gln Gln Lys Leu Glu Glu Gln Asn>
                                                                        C3'

          970         980         990        1000        1010        1020
           *           *           *           *           *           *
AAG ATT TCA GAA GCA AGT CGC AAA GGC CTT CGC CGT GAT TTA GAC GCA TCA CGT GAA GCT
Lys Ile Ser Glu Ala Ser Arg Lys Gly Leu Arg Arg Asp Leu Asp Ala Ser Arg Glu Ala>
Lys Ile Ser Glu Ala Ser Arg Lys Gly Leu Arg Arg Asp Leu Asp Ala Ser Arg Glu Ala>

AAG AAA CAA
Lys Lys Gln>
Lys Lys Gln>
```

Figure 12

M24-M5-M6-M19 MULTIVALENT HYBRID M PROTEIN WITH SHORT SUBUNITS (15 AA)

```
          10          20          30          40          50          60
           *           *           *           *           *           *
ATG GTC GCG ACT AGG TCT CAG ACA GAT ACT CTG GAA AAA GTA CAA GAA GGA TCC GCC GTG
Met Val Ala Thr Arg Ser Gln Thr Asp Thr Leu Glu Lys Val Gln Glu Gly Ser Ala Val>
                                                                BamHI  M5
 M24_____...
          70          80          90         100         110         120
           *           *           *           *           *           *
ACT AGG GGT ACA ATA AAT GAC CCG CAA AGA GCA AAA GAA GTC GAC AGA GTG TTT CCT AGG
Thr Arg Gly Thr Ile Asn Asp Pro Gln Arg Ala Lys Glu Val Asp Arg Val Phe Pro Arg>
                                                  SalI   M6_____...
         130         140         150         160         170         180
           *           *           *           *           *           *
GGG ACG GTA GAA AAC CCG GAC AAA GCA CGA CCA TGG AGA GTG CGT TAT ACT AGG CAT ACG
Gly Thr Val Glu Asn Pro Asp Lys Ala Arg Pro Trp Arg Val Arg Tyr Thr Arg His Thr>
                                        NcoI   M19_____....
         190         200
           *           *
CCA GAA GAT AAG CTA AAA AAA TAA
Pro Glu Asp Lys Leu Lys Lys
```

Figure 13

M24-M5-M6-M19-M3-M1-M18-M12 MULTIVALENT HYBRID M PROTEIN

```
          10          20          30          40          50          60
           *           *           *           *           *           *
ATG GTC GCG ACT AGG TCT CAG ACA GAT ACT CTG GAA AAA GTA CAA GAA GGA TCC GCC GTG
Met Val Ala Thr Arg Ser Gln Thr Asp Thr Leu Glu Lys Val Gln Glu Gly Ser Ala Val>
    M24_____...                                                  BamH1__M5____.
          70          80          90         100         110         120
           *           *           *           *           *           *
ACT AGG GGT ACA ATA AAT GAC CCG CAA AGA GCA AAA GAA GTC GAC AGA GTG TTT CCT AGG
Thr Arg Gly Thr Ile Asn Asp Pro Gln Arg Ala Lys Glu Val Asp Arg Val Phe Pro Arg>
                                                 Sal1___M6_____...
         130         140         150         160         170         180
           *           *           *           *           *           *
GGG ACG GTA GAA AAC CCG GAC AAA GCA CGA CCA TGG AGA GTG CGT TAT ACT AGG CAT ACG
Gly Thr Val Glu Asn Pro Asp Lys Ala Arg Pro Trp Arg Val Arg Tyr Thr Arg His Thr>
                                       NcoI___M19_____....
         190         200         210         220         230         240
           *           *           *           *           *           *
CCA GAA GAT AAG CTA AAA AAA CTG CAG GAT GCT AGG AGT GTT AAT GGA GAG TTT CCT AGA
Pro Glu Asp Lys Leu Lys Lys Leu Gln Asp Ala Arg Ser Val Asn Gly Glu Phe Pro Arg>
                            PstI___M3_____....
         250         260         270         280         290         300
           *           *           *           *           *           *
CAT GTT AAA TTA ATC GAT AAC GGT GAT GGT AAT CCT AGG GAA GTT ATA GAA GAT CTT GCA
His Val Lys Leu Ile Asp Asn Gly Asp Gly Asn Pro Arg Glu Val Ile Glu Asp Leu Ala>
                  ClaI___M1_____....
         310         320         330         340         350         360
           *           *           *           *           *           *
GCA GAA TTC GCA CCT CTT ACT CGA GCT ACA GCA GAC AAT AAA GAC GAA TTA ATA CGA TCG
Ala Glu Phe Ala Pro Leu Thr Arg Ala Thr Ala Asp Asn Lys Asp Glu Leu Ile Arg Ser>
    EcoR1__M18_____.....                                                 PvuI___
         370         380         390         400
           *           *           *           *
CAT AGT GAT TTA GTC GCA GAA AAA CAA CGT TTA GAA GAT TTA GGA TAA
His Ser Asp Leu Val Ala Glu Lys Gln Arg Leu Glu Asp Leu Gly ***>
M12_____.....
```

INTERNATIONAL SEARCH REPORT

Int...ational application No.
PCT/US93/08703

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 31/18, 39/085; C12N 1/21
US CL :424/92; 435/252.3, 253.4, 69.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/92; 435/252.3, 253.4, 69.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Chem Abstracts, Medline, EMBASE, Biosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,124,153 (Beachey et al) 23 June 1992, see column 3 lines 61-68 and column 8, lines 43-50.	1-9 and 12-20
Y	Vaccine, Volume 6, No. 2, Issued April 1988, Beachey et al, "Protective and Autoimmune Epitopes of Streptococcal M Proteins, pages 192-196, see pages 193 and 195.	3 and 6-9
Y	Journal of Experimental Medicine, Volume 163, No 6, Issued 01 June 1986, Beachey et al, "Opsonic Antibodies Evoked by Hybrid Peptide Copies of Types 5 and 24 Streptococcal M Proteins Synthesized in Tandem", pages 1451-1458, see page 1456.	1-9 and 12-20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 03 December 1993	Date of mailing of the international search report 16 DEC 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer JULIE KRSEK-STAPLES Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/08703

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Immunology, Volume 148, No. 3, Issued 01 February 1992, Bronze et al, "Epitopes of Group A streptococcal M protein that evoke cross-protective local immune responses", pages 888-893, see page 889.	12 and 20